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# Enzyme Inhibition in Relation to Chemotherapy.* (17313)

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Studies on enzyme inhibitors are frequently reported in terms of the percent inhibition in comparison with a control containing no inhibitor. It is the purpose of the present paper to show that this approach is frequently in danger of giving misleading results for the reason that with some inhibitors the percent inhibition is a function of the enzyme concentration. As will be pointed out in the discussion, the existence of such inhibitors is of considerable importance from the standpoint of pharmacology, chemotherapy and related fields, and it is therefore of some importance to have an effective experimental method for their recognition.

The phenomenon which we propose to describe is essentially the outcome of an "irreversible" reaction between enzyme and inhibitor, so that for all practical purposes the enzyme is effectively titrated or stoichiometrically combined with a definite amount of inhibitor. From the theoretical standpoint the "irreversible" reaction between enzyme and inhibitor may occur in a variety of ways; the simplest case is one in which the enzyme-inhibitor complex is theoretically reversible but has a dissociation constant so small that the combination seems irreversible. We shall therefore refer to this type of inhibition as *pseudo-irreversible*. Equally possible is a situation in which the enzyme reacts with an inhibitor in a truly irreversible manner, that is to say, the enzyme is converted to a form which cannot be converted back into active enzyme. In either case, the amount of enzyme inactivated will depend not only upon the amount of inhibitor but upon the amount of enzyme present. Regardless of the exact nature of the irreversibility, it can be recognized very simply by means of an experimental test: by determining the rate of reaction at different enzyme concentrations plus or minus inhibitor, it should be found that in the case of the controls, the rate is proportional to the enzyme concentration, so that a straight line through the origin is obtained when rate is plotted against enzyme amount. In the presence of a reversible inhibitor a straight line through the origin also results, but the slope of the line is less than in the case of the control. In the case of an irreversible inhibitor, the slope of the line is the same as that of the control but it will pass through the X-axis to the right of the origin by an amount that is proportional to the amount of inhibitor (see Bain[1]). This graphic test is not de-

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A preliminary report of this work was given at the Annual Meeting of the American Association for Cancer Research, April 16 and 17, 1949, (*Cancer Research*, 1949, **9**, 602).

[1] Bain, J. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 9.

pendent upon any theoretical assumptions as to the nature of the irreversibility and is of considerable value in the interpretation of inhibitor data whether obtained with pure enzymes, homogenates, minces, slices or whole cells. It is of interest that the curve for the pseudo-irreversible type of inhibitor is simply one of a family of curves, and that the curve for the reversible type can be obtained from the same general equation that is derived from the familiar Michaelis-Menten equation in the theoretical part of this paper. With the same equation it can be shown that when the dissociation constant of the enzyme-inhibitor complex has an intermediate value, the plot of activity against enzyme amount yields a line that is intermediate between the two types described above. Since many of the recently discovered enzyme inhibitors appear to fall in this category the test may be of value in understanding their action, since in these cases the per cent inhibition also depends upon the enzyme concentration.

The fact that inhibitors may fall into 3 broad categories depending upon the relationship between the dissociation constant of the enzyme-inhibitor complex and the concentration of enzyme has been emphasized previously by Straus and Goldstein[2] and by Goldstein.[3] They pointed out that previous mathematical treatments based on the Michaelis-Menten equation assumed "that the concentration of enzyme centers is constant and so small compared with the concentration of any substance with which it may combine that it may be neglected." They called attention to the fallacy in this assumption and developed the Michaelis-Menten equation along lines that include the factor of enzyme concentration.†

Our own observations and those of Bain[1] are in accord with the conclusions of Straus and Goldstein[2,3] and are believed to supplement their work by presenting a simple graphic method for recognizing the situations in which the older mathematical treatments are more or less inadequate. The graphic method is accompanied by a mathematical analysis in which one of the components of the graph (rate) is obtained as a function of the other component (enzyme amount) in an equation involving other variables (substrate amount, inhibitor amount, enzyme-substrate dissociation constant, and enzyme-inhibitor dissociation constant). The graphic method involves no assumptions as to the mechanism of inhibitor action, but in conjunction with the equation given it permits one to obtain an apparent dissociation constant for the enzyme-inhibitor complex. The word "apparent" is used here because in the case of an inhibitor which gives a line that intercepts the X-axis it would not be possible to decide from the graph whether the inhibitor was pseudo-irreversible (*i.e.*, dissociation constant of enzyme-inhibitor complex very small) or truly irreversible. In addition, the apparent dissociation constant will be affected by the specificity of the inhibitor and the presence of other compounds with which it may combine. It must be emphasized that the main purpose of the graph is not to determine the dissociation constants but to distinguish between the reversible type of inhibitor and the "irreversible" inhibitors. It is believed that the graph will permit this distinction regardless of the presence of interfering compounds and no evidence to the contrary has been obtained.

The data given are for illustrative purposes only, and have no intrinsic interest except insofar as they illustrate the principles described above. For this work, succinoxidase was employed as the test system because previous experience[4] showed that it could be

2 Straus, O. H., and Goldstein, A., *J. Gen. Physiol.*, 1943, **26**, 559.

3 Goldstein, A., *J. Gen. Physiol.*, 1944, **27**, 529.

† The important contributions by Michaelis and Menten, by Haldane, and by Lineweaver and Burk (referred to by Straus and Goldstein[2,3]) provide the foundation for the further expansion of the basic equations to include the amount of enzyme as a variable. The older equations are still very useful as well as appropriate in situations in which competitive inhibition between reversible inhibitors and substrates occurs since in these situations variation in enzyme amount does not affect the per cent inhibition. The newer treatment is more appropriate in the case of the pseudo-irreversible inhibitors.

inhibited by two types of inhibitors, the malonate-type and the type that combines with sulfhydryl groups. Whole homogenates were used as in the previous work, in which it was shown that the sulfhydryl inhibitors acted upon an essential group that could be protected by malonate, although malonate would not protect other enzymes against the sulfhydryl inhibitors. It thus seems clear that the sulfhydryl reagents used in this study inactivate succinoxidase by direct interaction with the enzyme.

*Experimental. Test System.* In these studies the succinoxidase system was employed. In all experiments a 10% water homogenate of liver was prepared in the usual manner and used as a source of the succinic dehydrogenase after diluting to 2.5%. The basal reaction mixture used was the same in all cases and prepared as previously described.[5] The concentration of inhibitors and homogenate are in the corresponding plots of the experimental data. The total volume of material in each flask was 3 ml and the temperature of the reaction 38°C. The rate of oxygen uptake per 10 minutes was determined on the basis of 4 successive 10-minute periods. With all inhibitors except oxalacetate in experiments reported in Fig. 1, the homogenate was incubated for 30 minutes at room temperature with the inhibitor before the addition of succinate, since some inhibitors do not react instantly with the enzyme, and are affected by the presence of the substrate.

*Compounds Tested.* The itaconic acid was obtained from the Chas. Pfizer Company while the malonic acid and quinone were obtained from the Eastman Kodak Company. Oxalacetic acid was prepared from sodium ethyl oxalacetate.

*Results. Effect of Enzyme Concentration.* The reaction velocity for the enzymatic dehydrogenation of succinate to fumarate could be measured in terms of the oxygen taken up in successive 10-minute intervals, since the succinic dehydrogenase is the limiting factor in the succinoxidase system when both cytochrome c and cytochrome oxidase are present in excess. It was established that the reaction rate in the absence of inhibitors was proportional to the amount of homogenate. Since a different liver homogenate was used for each experiment, it was necessary to include controls each time an inhibitor was tested. It may be seen in Fig. 1 that the control data in each instance (open circles) yielded a satisfactorily straight line that passes through the origin.

However, when such data were obtained with various inhibitors present and were plotted in the same manner, significant differences between the inhibitors were revealed insofar as they were affected by enzyme concentration.

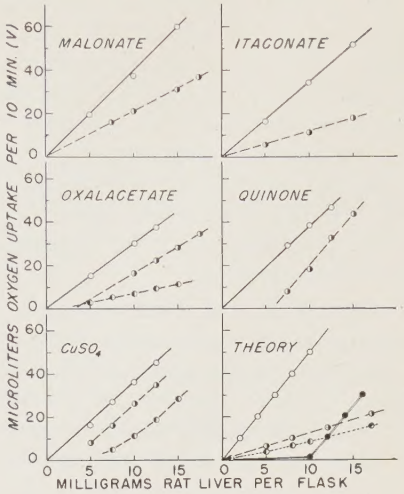


FIG. 1.

The reaction velocity as measured by oxygen uptake for the enzymatic dehydrogenation of succinate to fumarate in the presence and absence of inhibitors. The concentration of malonate was $1 \times 10^{-3}$M; oxalacetate was $6.7 \times 10^{-5}$M and $3.4 \times 10^{-5}$M; copper sulfate was $1.3 \times 10^{-4}$M and $2.6 \times 10^{-4}$M; itaconate was $5 \times 10^{-2}$M; quinone was $3.3 \times 10^{-4}$M. In all cases the concentration of succinate was $5 \times 10^{-2}$M. In the curve systems labeled malonate, oxalacetate, Cu $SO_4$, itaconate and quinone, the broken lines represent activity in the presence of the inhibitors. The system of curves labeled theory were calculated from equation (13) assuming for all cases $S = 5 \times 10^{-2}$M and $K_s = 10^{-3}$: for the solid curve $I_t = 0$; for the broken curve $K_i = 10^{-4}$, $I_t = 1 \times 10^{-3}$M; for the dashed curve $K_i = 10^{-6}$, $I_t = 5 \times 10^{-5}$M; for the double lined curve $K_i = 10^{-9}$, $I_t = 1 \times 10^{-5}$M.

[4] Potter, V. R., and Du Bois, K. P., *J. Gen. Physiol.*, 1943, **26**, 391.

[5] Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, **149**, 217.

In the presence of malonate or itaconate (Fig. 1), which are structurally similar to succinate, the rate of dehydrogenation was also proportional to the enzyme concentration, but the slope of the line was less than in the case of the controls. The per cent inhibition was thus the same at any concentration of enzyme and depended solely upon the concentration of inhibitor since the succinate concentration was constant.

In contrast to the data obtained with malonate and itaconate, oxalacetate, which also bears a close structural relationship to succinate, yielded data that do not fall on a line through the origin, but rather on a line which appears to intercept the enzyme axis (Fig. 1).

Even more striking are the results with quinone and copper (Fig. 1). Here the data fell on lines that tend to be straight and parallel to the control. With these inhibitors the per cent inhibition is clearly related not only to the inhibitor concentration but also to the enzyme concentration.

*Interference Phenomenon with Irreversible Inhibitors.* In earlier studies on succinoxidase[4] it was shown that the inhibition produced by malonate was instantaneous and independent of the order of addition of substrate and inhibitor. This was in contrast to results obtained with agents like quinone and copper, which produced a slowly increasing per cent inhibition that was strongly affected by the presence or absence of succinate or, indeed, of malonate. In the presence of succinate or malonate, the sulfhydryl inhibitors react more slowly with the enzyme than they do when both of these compounds are absent. A more detailed experiment on this point is reported in Fig. 2. In this experiment a series of Warburg flasks was prepared with the usual reaction mixture but no succinate, and with an equal amount of tissue in each flask. At zero time copper in the form of $CuSO_4$ was added to all of the flasks, and at successive intervals succinate was added from the side arm and oxygen uptake was measured. The continuous lines represent the rate of oxygen uptake during successive 10-minute time intervals after the

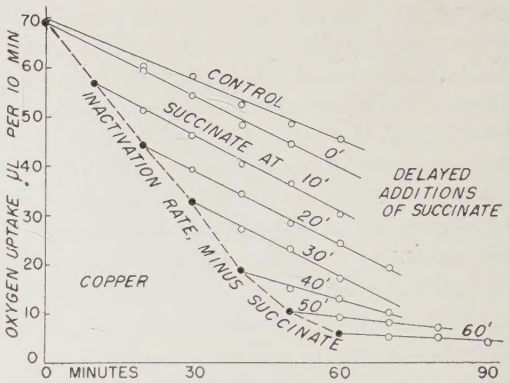


FIG. 2.

Rate of inactivation of succinic dehydrogenase by copper in the presence and absence of succinate. The final concentrations of succinate and of Cu $SO_4$ were 0.067 M and 3.3 $\times$ $10^{-5}$M, respectively. The solid circles represent extrapolated points. The open circles are experimental points.

addition of succinate, and show that when succinate was present no inactivation of the enzyme by the copper occurred, since the decline in rate was no greater than in the controls. The broken line indicates the rate of enzyme inactivation by copper when no succinate was present, and shows that a progressive decrease in active enzyme occurred as long as succinate was absent, but as soon as succinate was added the process of inactivation ceased abruptly. This experiment shows that succinate *interferes* with the reaction between copper and succinic dehydrogenase, while copper once combined with the enzyme cannot be effectively displaced by succinate. When larger amounts of sulfhydryl inhibitors were added to the succinoxidase system, it was possible to progressively inhibit the enzyme even in the presence of succinate, although not as rapidly as in the absence of succinate. It is clear that copper and succinate compete for the enzyme and that their reactions with it are *mutually exclusive* as suggested earlier in the case of quinone.[4] For the present we shall refer to this type of effect as an "interference phenomenon" and will not attempt to describe it as competitive inhibition. But it must be pointed out that the latter term is a purely operational concept that is at present restricted to reversible inhibitors. The experimental approach indi-

cated in Fig. 2 provides a means of studying competition between substrates and irreversible inhibitors during the course of the reaction between the inhibitor and the enzyme as opposed to competition during the measurement of enzyme activity. It is proposed that this kind of competition be called *interference* in order to avoid needless confusion in terminology.

*Theoretical.* To derive an expression in which ES is expressed as a function of $E_t$, S, $I_t$, $K_s$, and $K_i$, the symbols listed below were used:

$E_t$ = Total Enzyme Concentration.
$E_f$ = Uncombined Enzyme Concentration.
EI = Enzyme-Inhibitor Complex Concentration.
ES = Enzyme-Substrate Complex Concentration.
S = Total Substrate Concentration.
$S_f$ = Uncombined Substrate Concentration.
P = Product Concentration.
$K_i$ = Enzyme-Inhibitor Complex Dissociation Constant.
$K_s$ = Enzyme-Substrate Complex Dissociation Constant.
$I_t$ = Total Inhibitor Concentration.
$I_f$ = Uncombined Inhibitor Concentration.
k = Velocity Constant.

The equation was developed along lines similar to the familiar Michaelis-Menten equation but includes more variables.† The following assumptions were made:

(a) The reaction between enzyme and substrate or inhibitor can be formulated as in equations (1) and (2):

$$E_f + S_f \rightleftharpoons ES \longrightarrow P + E_f \qquad (1)$$
$$E_f + I_f \rightleftharpoons EI \qquad (2)$$

(b) Where (S) is very large, $(S_f)$ is assumed to equal (S) and $(E_f)$ approaches zero.

(c) The total inhibitor concentration is equal to the sum of the uncombined and combined forms.

$$(I_t) = (I_f) + (EI) \qquad (3)$$

(d) The total enzyme concentration is the sum of the uncombined enzyme, that combined with the inhibitor and that combined with the substrate

$$(E_t) = (E_f) + (EI) + (ES) \qquad (4)$$

An equation in which ES is expressed as a function of $E_t$, S, $I_t$, $K_s$ and $K_i$ may now be derived.

From expressions (1) and (2) and the mass law one can write:

$$\frac{(E_f)\quad(S_f)}{(ES)} = K_s \qquad (5)$$

$$\frac{(E_f)\quad(I_f)}{(EI)} = K_i \qquad (6)$$

By substituting the value of (EI) from (6) into (3) and rearranging one obtains expression (7).

$$(I_f) = (I_t)/\left[1 + \frac{(E_f)}{K_i}\right] \qquad (7)$$

If one substitutes $(I_f)$ of expression (7) into (6), solves the resulting expression for (EI) and substitutes what is then equivalent to (EI) into (4) one arrives at expression (8).

$$(E_t) = (E_f) + \frac{\dfrac{(E_f)\quad(I_t)}{K_i}}{1 + \dfrac{(E_f)}{K_i}} + (ES) \qquad (8)$$

Since from (b), $(E_f)$ is small, (8) reduces to (9)

$$(E_t) = \frac{\dfrac{(E_f)\quad(I_t)}{K_i}}{1 + \dfrac{(E_f)}{K_i}} + (ES) \qquad (9)$$

If one solves equation (5) for $(E_f)$ and substitutes the result into (9) one arrives at (10).

$$(E_t) = \left[\frac{(I_t)}{1 + \dfrac{K_s\ (ES)}{K_i\ (S)}}\right]\left[\frac{K_s\ (ES)}{K_i\ (S)}\right] + (ES) \qquad (10)$$

By multiplying through equation (10) by the term $1+[K_s\ (ES)]/[K_i(S)]$ and rearranging and solving for (ES) by the quadratic formula one obtains expression (11), in which (ES) is expressed as a function of $(E_t)$.

$$(ES) = -\left[\frac{K_i\ (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right] \pm \sqrt{\left[\frac{K_i\ (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right]^2 + \frac{K_i\ (S)\quad(E_t)}{K_s}} \qquad (11)$$

Since the velocity of reaction (V) is equal to the product of the concentration of the enzyme-substrate complex and the velocity constant for its conversion to the product, then:

$$V = k\ (ES) \qquad (12)$$

or if one multiplies through expression (11) by k and substitutes $V = k\ (ES)$ one obtains‡

$$V = -k\left[\frac{K_i\ (S)}{2(K_s)} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right] \pm k\sqrt{\left[\frac{K_i\ (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right]^2 + \frac{K_i\ (S)\ (E_t)}{K_s}} \qquad (13)$$

The velocity is thus expressed in terms which can be experimentally determined.

We may now consider various tests of the equation. If one lets $(I_t)$ equal O, then equation (13) reduces to the following form:

$$V = \frac{-k(S)\ K_i}{2K_s} + \frac{k(E_t)}{2} + \sqrt{\frac{k(S)\ K_i}{2K_s} + \frac{k(E_t)}{2}}$$

$$V = k(E_t) \qquad (14)$$

For large values of (S) and no inhibitor, it follows from expression (4) that $(E_t)$ equals $(E_s)$; hence expression (14) is in agreement with (12). Expression (14) is the equation of a straight line of slope k passing through the origin. It is also what is experimentally observed when values of V are plotted against corresponding values of $(E_t)$ in the absence of inhibitor (Fig. 1). The slope is the velocity constant for conversion of the enzyme-substrate complex to the product.

For small values of $K_i$, *i.e.* where the binding of inhibitor with enzyme is great compared to that of the substrate with enzyme, the terms of equation (13) containing $K_i$ approach zero and the equation approaches the form of a straight line with a slope k and an intercept on the enzyme axis. Such an inhibitor virtually "titrates" the enzyme.

$$V = \frac{-k}{2}\left[(I_t) - (E_t)\right] \pm k\sqrt{\left[\frac{(I_t) - (E_t)}{2}\right]^2}$$

$$V = k(E_t) - k(I_t) \qquad (15)$$

However equation (14) only approaches the form of (15) since the term containing $K_i$ only approaches zero. When equation (14) is plotted using various values of $K_i$, a system of curves is obtained as shown in Fig. 1, theoretical curves. For values of $K_i$ that approach the magnitude of $K_s$, *i.e.* $10^{-4}$ compared to $10^{-2}$, a straight line through the origin is obtained. For smaller $K_i$ values a curved line is obtained and for very small values, *i.e.*, $10^{-9}$, a curve is obtained which rapidly approaches a straight line at a point equal to $kI_t$ and has a slope equal to k.

These plots may assist in the interpretation of the experimental data. Malonate with a $K_i$ equal to $10^{-4}$ gives a straight line through the origin. Itaconate, a less effective inhibitor, is of the same nature. Copper and quinone which combine with sulfhydryl groups probably have very small $K_i$ values, while oxalacetate,[6] an intermediate case ($K_i$ near $10^{-6}$), occupies an intermediate position.

Thus it is possible to explain the nature of the experimental curves relating velocity of reaction to enzyme concentration on the basis of the degree of binding of the enzyme-inhibitor complex relative to the enzyme-substrate complex. This approach also provides an experimental method for determining when one is justified in using the Michaelis-Menten equation to calculate values of $K_i$ or when a more exact expression is necessary.

‡ On the basis of the same assumption made in deriving equation (11), Goldstein[3] has developed the following expression:

$$I' = S'\left[\frac{1-a}{a}\right] + (1-a)\ E_i'$$

wherein I', S' and E' represent the "specific concentrations" of I, S and $E_t$ and a is the fractional activity of the enzyme. If one multiplies through the expression of Goldstein by the dissociation constant, $K_i$, of the enzyme-inhibitor complex, converts the specific concentrations to absolute concentrations and substitutes $ES/E_t$ for a, then on solving the resulting equation for (ES) in terms of $I_t$, $K_i$, $K_s$ and $E_t$ one also arrives at expression (11) which was used in developing expression (13).

[6] Pardee, A. B., and Potter, V. R., *J. Biol. Chem.*, 1948, **176**, 1085.

If a plot of data representing the change of velocity of reaction with changes in enzyme concentration appears to intercept the enzyme axis when extrapolated to zero velocity, then the amount of inhibition will vary with enzyme concentration. In such a case the value of $K_i$ determined from the Michaelis-Menten equation will not be a constant but will vary depending on the enzyme concentration used in the experiment and a more exact expression than the Michaelis-Menten equation is necessary for calculating $K_i$. We have not attempted to derive such an expression because in the case of "titration type" curves the results can be due either to high affinity or true irreversibility as explained earlier.

The analysis represented by equation (11) is incomplete insofar as it does not encompass the effect of the presence of substances other than the enzyme that can combine with the inhibitor. It is clear that the more specific is the inhibitor the less important these considerations will be.

*Discussion.* The main purpose of this paper is (1) to show that in the case of "irreversible" inhibitors, regardless of the nature of the irreversibility, the degree of enzyme inhibition depends upon the enzyme concentration and (2) to consider the implications of this fact. The first question that arises is whether the phenomenon of decreasing per cent inhibition with increasing enzyme amount is a general phenomenon for high affinity inhibitors, or whether the data presented here are merely due to the fact that the high affinity inhibitors are non-specific and are used in a whole homogenate. We believe that the phenomenon is a general one and that increasing numbers of examples will be found (*cf.* Bain[1]).¶ It is difficult to see how the presence of the other SH groups in the homogenate could produce the type of curve seen with copper or quinone in contrast to the malonate curve, since the ratio between succinoxidase SH groups and "other" SH groups would remain constant as the amount of homogenate was increased. The enzyme system and inhibitors chosen for this study are probably not the best possible examples for illustrative purposes, but the data and conclusions are strongly supported by the accompanying paper on cholinesterase by Bain.[1] In the case of any "irreversible" inhibitor the degree of inhibition produced at any given molarity will depend upon the concentration of enzyme *and* upon the concentration of other substances that will combine with the inhibitor. This fact applies to any inhibitor, since it can never be assumed that an inhibitor, even though it is known to be highly specific, does not react with unknown constituents in living cells or in preparations therefrom. The reaction with unknown constituents becomes of great importance if it is irreversible. However, the occurrence of side reactions does not invalidate the conclusion that copper and quinone in effect irreversibly inactivate succinoxidase. This inactivation cannot be reversed by succinate, (which has a lower affinity for the *enzyme* than copper does) but can be reversed by glutathione,[7] which has an affinity for *copper* similar to that of the enzyme (cf also Barron and Kalnitsky.[8]) This inhibition is therefore considered to be pseudo-irreversible, *i.e.* it has a dissociation constant, but the constant is so small that the percent inhibition varies with the amount of enzyme.

Oxalacetate, which is an intermediate case, is a potent inhibitor for succinoxidase and is not known to inhibit any other enzyme (its action in the malic dehydrogenase system is an equilibrium effect.)

It is thus concluded that the results are not artifacts and that their significance may be examined.

From the standpoint of studies in enzyme

¶ Note added Oct. 24, 1949: Since this paper was submitted for publication, two additional reports that the enzyme concentration may affect the degree of inhibition have appeared: E. C. Slater reported studies with a partially purified succinoxidase system in *Biochem. J.*, 1949, **45**, 130, especially page 138, and O. H. Lowry, O. A. Bessey and E. J. Crawford have reported the effect of 2-amino-4-hydroxy-6-formylpteridine upon pterine oxidase and commented that "The extremely low dissociation of the enzyme-inhibitor complex permits a virtual titration of the enzyme." (*J. Biol. Chem.*, 1949, **180**, 399).

[7] Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, 1938, **32**, 1829.

[8] Barron, E. S. G., and Kalnitsky, G., *Biochem. J.*, 1947, **41**, 346.

kinetics as carried out *in vitro,* it appears that experiments along the lines suggested by Fig. 1 and 2 would be helpful in the study of any new inhibitor. If the plot of rate against enzyme concentration is a straight line through the origin in the presence of inhibitor (*cf* malonate, Fig. 1) and if the inhibition is independent of the time of addition of substrate (in contrast to Fig. 2) then it is feasible to proceed with the determination of $K_i$ values and tests for competitive inhibition. On the other hand, if the data are comparable to those obtained with copper, quinone, or oxalacetate, reports of inhibitor potency without showing the effect of enzyme concentration will be meaningless while the determination of $K_i$ values and the tests for competitive inhibition by existing methods will also be of little significance.

It is from the standpoint of studies on whole animals or on tissue preparations that the results have the greatest significance. If it is established that the "irreversible" inhibitors (either pseudo-irreversible or irreversible) affect enzyme activity to an extent that depends upon the concentration of the enzyme and the concentration of inhibitor-binding compounds in the tissue, it may be anticipated that when an inhibitor (or drug or chemotherapeutic agent) is injected into an animal different tissues and tissue components will be inhibited to different extents depending on their composition. In other words, a tissue containing a small amount of a given enzyme might have its enzyme completely inactivated by an injection of an inhibitor that would inactivate only a small fraction of the enzyme in tissues containing larger amounts of enzyme and comparable amounts of other reacting components. Data which may be an illustration of this phenomenon are available in a paper by DuBois and Mangun.[9] Following injections of hexaethyl tetraphosphate at a level of 1 mg/kg in rats, they found that the per cent inhibitions of acetyl-choline esterase were 4.5, 22 and 100 in samples of brain, submaxillary gland and serum respectively. If these variations are to be explained as a result of variations in the amount of cholinesterase in the 3 tissues, one would expect the enzyme content of these tissues to vary in the way that was actually observed, *viz.* the authors found relative activity values of 100, 28 and 10 in the 3 tissues from control animals. Thus the absolute amount of enzyme destroyed was very similar in the 3 tissues, *i.e.,* 4.5, 6.2 and 10.0. Injections of higher levels of inhibitor produced 100 per cent inhibition first in submaxillary gland, then in brain. These data do not prove the point that is being made here since many factors obviously affect the data in the case of whole animals; but the viewpoint stressed here may need to be included in any future examination of similar data.

The production of alloxan-diabetes[10] may be cited as another possible example of this type of phenomenon, with the difference that in this case specific cells have been destroyed without the demonstration of inhibition of an enzyme. Alloxan is a general SH inhibitor and it seems very unlikely that it reacts with an enzyme occurring only in the insulin-producing cells. According to our interpretation, these cells would be more vulnerable to alloxan because of smaller amounts of a vital enzyme that is more plentiful in other tissues. The specific destruction of insulin-producing cells by alloxan may serve as a prototype for cancer chemotherapy. Since the enzyme pattern in cancer tissue may conceivably include no enzymes that are not also found in normal tissues (*i.e.* if it arises by a process of enzyme deletion), the chemotherapy of cancer may require the inhibition of an enzyme that is also present in normal tissues. It should be possible to completely inhibit an enzyme present in cancer tissue in small amounts, while producing only partial inactivation of the enzyme in tissues containing it in larger amounts.

The interference phenomenon described in Fig. 2 is of considerable importance in interpreting studies on whole animals, especially since interference between reversible and ir-

[9] Du Bois, K. P., and Mangun, G. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 137.

[10] Lukens, F. O. W., *Physiol. Rev.*, 1948, **28**, 304.

reversible inhibitors can occur.[4] Koster[11] found that diisopropylfluorophosphate (DFP) given before physostigmine made animals more *sensitive* to the latter, while injections of the latter protected animals against doses of DFP that were several-fold greater than lethal. These data can be explained in terms of interference between the two inhibitors plus the fact that the DFP can be destroyed in the body as shown by Mazur.[12] In other words, physostigmine would lower the concentration of free enzyme and protect it against DFP for a time sufficient to allow for the destruction of DFP, while DFP initially would lower the effective concentration of enzyme thereby sensitizing the animal against physostigmine.

*Summary.* 1. The effect of enzyme concentration on the inhibition produced by certain inhibitors of the succinic dehydrogenase system has been experimentally determined. The inhibitors studied were malonate, itaconate, oxalacetate, quinone and cupric ion.

2. The reaction between succinic dehydrogenase and copper or quinone was not immediate but required 30 to 40 minutes when the amount of inhibitor was just sufficient to produce complete inhibition.

3. The effect of the strengths of binding of the inhibitor with the enzyme on the per cent inhibition produced is shown to be related to the enzyme concentration.

4. An expression has been developed which relates the velocity of reaction to enzyme concentration in enzyme-inhibitor systems.

5. The results are discussed in relation to the chemotherapy of cancer. It is pointed out that the selective inhibition of an enzyme not unique to cancer tissue is theoretically possible.

The authors gratefully acknowledge the helpful suggestions of Dr. J. A. Bain, whose data on cholinesterase inhibition were made available to us prior to publication.

11 Koster, R., *J. Pharmacol.*, 1946, **88**, 39.

12 Mazur, A., *J. Biol. Chem.*, 1946, **164**, 271.

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## Mechanism of the Inhibition of Rat Brain Cholinesterase by Diisopropylfluorophosphate, Tetraethylpyrophosphate, and Eserine.* (17314)

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Kinetic analysis of the inhibition of enzyme systems has generally been carried out by methods which assume a reversible combination of the inhibitor and enzyme to form an inactive complex.[1–4] Ordinarily, dissociation constants for the enzyme-inhibitor complex are calculated according to the classical Michaelis-Menten treatment[1] from the concentration of inhibitor at which enzymatic activity is reduced 50 per cent. Goldstein[5] and Ackermann and Potter[6†] have pointed out that situations may exist when the assumptions used in the Michaelis-Menten derivation are no longer valid. Such a case arises when

* Supported in part by grants from the Miller Epilepsy Fund, the Rockefeller Foundation, and the Research Council of the Scottish Rite Masons.

1 Michaelis, L., and Menten, M. L., *Biochem. Z.*, 1913, **49**, 1333.

2 Haldane, J. B. S., Enzymes, Longmans, Green & Co., London, 1930.

3 Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, 1934, **56**, 658.

4 Ebersole, E. R., Guttentag, C., and Wilson, P. W., *Arch. Biochem.*, 1944, **3**, 399.

5 Goldstein, A., *J. Gen. Physiol.*, 1944, **27**, 529.

6 Ackermann, W. W., and Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 1.

† The author is indebted to Dr. V. R. Potter for making available a manuscript of this paper[6] prior to publication and for much correspondence and discussion regarding its contents.

the enzyme-inhibitor complex formation is irreversible (*i.e.*, the dissociation constant approaches zero) in which case the actual concentration of the enzyme may no longer be ignored. However, such irreversibility is not always plainly apparent and when independent evidence of such a state of affairs is lacking an investigator may analyze his data by the classical methods referred to above and arrive at values for dissociation constants that will be theoretically unsound. It is not always easy to devise unequivocal tests for irreversibility particularly when working with unpurified enzyme preparations such as serum, homogenates, minces, slices or tissue extracts. Ackermann and Potter[6] have recently proposed a method which offers a simple, graphical solution to this difficulty. During the course of a study of the effects of convulsant and anticonvulsant drugs on the cholinesterase system of rat brain tissue[7] we have had occasion to test the method of Ackermann and Potter using the newly discovered cholinesterase inhibitors diisopropylfluorophosphate and tetraethylpyrophosphate for which independent evidence for irreversibility exists[8] and compared their mechanism of action to that of the classical inhibitor, eserine (physostigmine).

*Experimental methods and results.* The general characteristics of the cholinesterase system in rat brain homogenates and the methods for its study have been previously described.[7] The specific conditions used in this investigation are given in table headings and figure legends.

The degree of inhibition by a given concentration of DFP or TEPP‡ is dependent upon the time of incubation of the inhibitor with the enzyme before addition of the substrate, as may be seen from the curves given in Fig. 1. In subsequent experiments we have arbitrarily adopted the time of 40 minutes incubation of the enzyme with inhibitor before addition of substrate. The enzyme may be protected from TEPP by addition of substrate simultaneous to, or before, the addition of the inhibitor (Table I). This effect is not so marked using DFP (Table I).

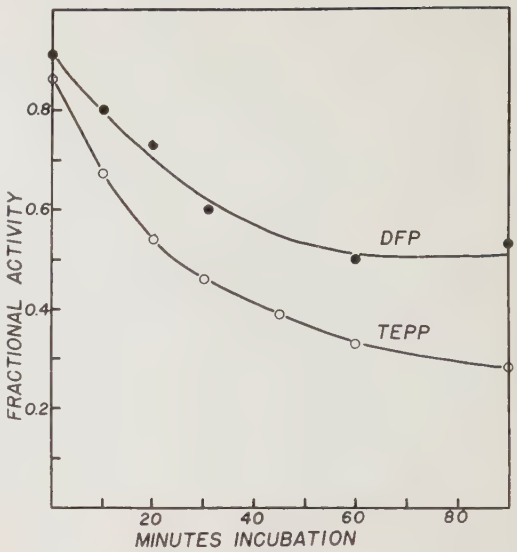


FIG. 1.

Effect of Incubation of the Enzyme with Inhibitor Before Addition of Substrate. Total volume 2.2 ml containing final concentrations as follows: 0.025 M $NaHCO_3$, 0.075 M KCl, 0.075 M NaCl, 0.04 M $MgCl_2$, 0.01 M acetylcholine-bromide (added from sidearm after incubation times indicated), $2.3 \times 10^{-7}$ M DFP or $8 \times 10^{-9}$ M TEPP, 25 mg (wet weight) per flask rat brain tissue added as 5% homogenate in buffer (see [7]). 5% $CO_2$—95% $N_2$ gas phase, temperature 37.5°C. Each point average of 2 experiments in duplicate. Activities calculated as in [7].

The inhibition of rat brain cholinesterase by DFP, TEPP and eserine under specified conditions is shown in the curves of Fig. 2 where enzyme activity is plotted against inhibitor concentration. It may be seen that the general shape of the curves is the same and without closer analysis it might be inferred that the inhibitors differed only in potency.

The results obtained using the method of Ackermann and Potter[6] are shown in Fig. 3. Here both tissue concentration and inhibitor concentration were varied. In the case of

[7] Bain, J. A., *Am. J. Physiol.*, in press.

[8] Bodansky, O., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 521.

‡ The abbreviations DFP—diisopropylfluorophosphate, and TEPP—tetraethylpyrophosphate, will be used throughout this paper. These compounds were obtained through the courtesy of Dr. H. E. Himwich of the Medical Division, Army Chemical Center, Edgewood, Md., and Dr. K. P. DuBois of the University of Chicago Toxicity Laboratory, Chicago, Ill.

TABLE I.
Effect of Order of Addition of Substrate and Inhibitor on Rat Brain Cholinesterase Activity. Basic conditions as in Fig. 1.

| Order of addition | % inhibition | |
|---|---|---|
| | TEPP ($8 \times 10^{-7}$ M) | DFP ($4 \times 10^{-6}$ M) |
| Simultaneous | 9 | 40 |
| Substrate 10′ before inhibitor | 2 | — |
| Inhibitor 30′ before substrate | 47 | 80 |

TEPP, when tissue concentration was plotted against rate for various concentrations of inhibitor a series of curves resulted, the control curve intercepting the x-axis at zero and the curves to which various amounts of TEPP had been added intercepting the x-axis at progressively larger values of tissue concentration. When these intercepts were plotted against the concentration of inhibitor (inset Fig. 3) a straight line through the origin was obtained. The same result was found with DFP as with TEPP, the former requiring higher concentrations, *e.g.*, the molarity at an x-intercept of 0.25 ml being 11.5 x $10^{-7}$ M DFP compared to 4.5 x $10^{-9}$ M TEPP. However, when the same procedure was applied to eserine a different result was obtained. It may be seen from Fig. 3, that in the case of eserine, a series of curves of different slopes results all intercepting the x-axis at zero in contrast to the DFP and TEPP curves.

Neither the eserine, the DFP, nor the TEPP inhibition of brain cholinesterase was reversed by cysteine as were the effects of the nitrogen mustards.[7] Atropine, which is a specific antidote for eserine and DFP *in vivo*, does not reverse their effects upon cholinesterase. Atropine itself does not affect the enzyme.[7] Eserine has been reported to protect brain cholinesterase against irreversible inactivation by DFP[9] and we have confirmed this finding.

*Discussion.* The values for the concentration required to give 50% inhibition of brain cholinesterase by DFP, TEPP, and eserine are of the same order of magnitude as those reported by others[10-12] using somewhat similar conditions; but it is apparent from the data in this report and that of others, particularly Nachmansohn, *et al.*,[13] that conditions of enzyme concentration, incubation time, and order of addition of inhibitor and substrate can profoundly affect the results with the fluorophosphates.

Eserine was included in this study because it and prostigmine are the only other compounds whose potency as inhibitors approaches that of the alkylated fluoro- and pyrophosphates.[14] The data presented in Fig. 3 clearly show that, under the conditions employed in this study, there is a difference in the nature of the inhibitory processes. Accepting the criteria of Ackermann and Potter,[6] DFP and TEPP are seen to fall into that category of inhibitors where analysis by the classical methods will no longer apply. Further, the irreversibility of the enzyme-inhibitor complex is demonstrated by a method which does not involve dialysis,[8] dilution,[13] nor isolation.[15] In the case of eserine, on the other hand, the data of Fig. 3 show that analysis of its inhibitory action may be successfully performed by the classical methods.

On the basis of the data in Fig. 3 and other independent criteria[8,13] the inhibition of cholinesterase by DFP and TEPP may probably be assumed to be irreversible. We may then consider that the inhibitor "titrates" a

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14 Augustinsson, K. B., *Acta Physiol. Scandinavica*, 1948, **15**, Suppl. 52.

15 Jansen, E. F., Nutting, M. D. F., and Balls, A. K., *J. Biol. Chem.*, 1949, **179**, 201.

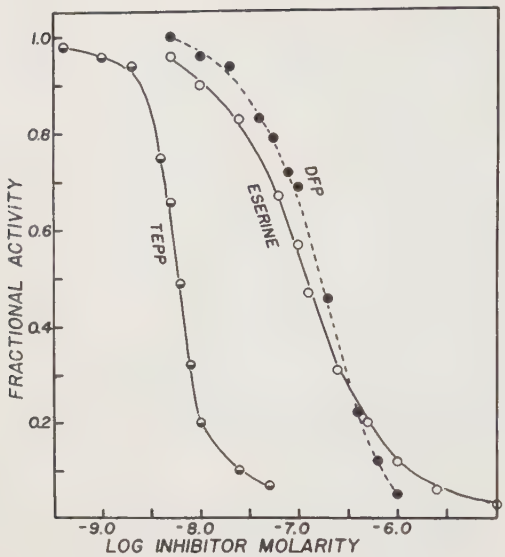


FIG. 2.

Inhibition of Rat Brain Cholinesterase by DFP, TEPP, and Eserine. Basic conditions as in Fig. 1. Inhibitors incubated with the enzyme 40 minutes before addition of substrate. Each point average of at least 2 experiments in duplicate.

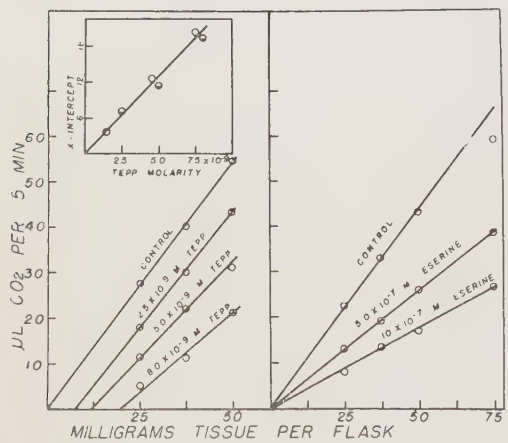


FIG. 3.

Effect of Variation in Enzyme Concentration on the Inhibition of Rat Brain Cholinesterase by TEPP and Eserine. Conditions as in Fig. 2. Inset figure: Half-solid circle - - points plotted from data given in this figure, ○ - - points plotted from another duplicate experiment using different rat brain as tissue source, included to show reproducibility.

certain amount of the enzyme, evaluated by the x-intercepts in Fig. 3. If we use the most potent inhibitor, TEPP, as the limiting case, it takes of the order of 1 x $10^{-11}$ moles of inhibitor to titrate the enzyme in 0.25 ml of homogenate which is equivalent to 0.0125 ml of original tissue. If we further assume that 1 mole of inhibitor combines with 1 mole of enzyme[§] then we may calculate that the greatest concentration of enzyme is approximately 1 x $10^{-9}$ moles per ml of tissue or 1 x $10^{-6}$ molar. The above reasoning disregards, of course, the possibility that the enzyme may be concentrated in certain regions by structural restrictions. Goldstein[5] has estimated that the concentration of cholinesterase in 4.5% dog serum has a maximum value of 2 x $10^{-8}$ M giving a value of approximately 1 x $10^{-7}$ M in 100% serum by direct extrapolation. We do not believe that the data in this paper justify any but the approximations indicated above because of considerable uncertainty as to the purity of the inhibitors used, the large dilutions of the inhibitor stock which are necessary and the possibility that some of the inhibitor is combined with proteins inert to the substrate employed.[‖] The calculations do serve, however, to give an upper limit to the enzyme concentration.

*Summary.* The mechanism of inhibition of rat brain cholinesterase by the 3 most potent anti-cholinesterases, diisopropylfluorophosphate, tetraethylpyrophosphate, and eserine was studied by a method which involves variation in enzyme concentration as well as in-

§ A reasonable assumption considering that Goldstein[5] found 1 mole of eserine per mole of enzyme and Jansen *et al.*[15] have recently shown that 1 mole of DFP combines with 1 mole of chymotrypsin to form an inactive complex. We have been unable to estimate, in the case of TEPP, the number of molecules of inhibitor per molecule of enzyme by the method of Goldstein because the slope of the inhibition curve (Fig. 2) falls above his theoretically determined limits, possibly because we are dealing here with an irreversible combination.

‖ This last consideration is somewhat discounted by the experiments of Mazur and Bodansky,[16] who added large amounts of heat-inactivated protein to a DFP-inhibited system and found no effect. However, the very process of heat inactivation may have destroyed groupings which in the native state were inert to the substrate but might not have been inert to the inhibitor.

16 Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, **163**, 261.

hibitor concentration. It was shown that the kinetics of eserine-inhibition may be analyzed by classical methods which ignore enzyme concentration but the cases of the other two inhibitors may not be so treated. Cholinesterase inhibition by the fluoro- and pyrophosphates was shown to be irreversible and to depend upon enzyme concentration and time of incubation of the enzyme with the inhibitor before the addition of substrate. The upper limit of cholinesterase concentration in rat brain was estimated to be $1 \times 10^{-6}$ molar if structural restrictions are not assumed.

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## Some Effects of Large Doses of Ergot Products on Rats.* (17315)

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An examination of the literature reveals much interest in various ergot products, particularly the dihydrogenated derivatives, because of their possible usefulness in the treatment of migraine,[1,2] in the prevention of cyclopropane arrhythmias,[3] and in the treatment of hypertension.[4–6]

Toxicity of the dihydrogenated derivatives is less than that of the natural alkaloids;[7] Orth and others[8] show that dihydroergocornine did not produce gangrene in the tails of rats, whereas ergotamine routinely produced gangrene. They also show that pregnant female rats receiving dihydroergocornine delivered normal litters and raised them to maturity, whereas similar rats receiving ergotamine tartrate lacked maternal instincts.

No accounts were found in the literature of experiments in which ergot products were injected over extended periods of time. Orth and others[8] made semiweekly injections during the gestation period of rats in doses up to 35 mg/kg of dihydroergocornine. Observations over extended periods were thought to be desirable since many patients would take the ergot product more or less regularly for years.

*Methods.* A total of 73 rats were injected subcutaneously 6 times a week for periods up to 17 weeks. This rigorous treatment contrasts with that of human therapy in which ergot products are usually injected only twice a week.

Control rats were injected 6 times a week with physiological saline.

All rats were weighed once a week.

Preliminary experiments show that doses comparable to therapeutic doses in man (for those products whose therapeutic doses have been established) produced no measurable effect on weight of rats. Therefore, it was decided to make the experiments still more rigorous by using doses comparable, on a weight basis, to those of ergotamine tartrate that produce gangrenous tails in rats. Preliminary experiments showed that a dose of about 0.5 mg/kg of ergotamine tartrate adminis-

* This study was made possible by a grant from the Sandoz Chemical Works, Inc.; the drugs were supplied by Mr. Harry Schnizer of that company.

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2 Marcussen, R. M., and Wolff, H. G., *J.A.M.A.*, 1949, **139**, 198.

3 Orth, O. S., *Arch. internat. de pharmacodyn. et de therap.*, 1949, **78**, 163.

4 Kappert, A., Baumgartner, P., and Rupp, F., *Schweiz. med. Wchnschr.*, 1948, **78**, 1265.

5 Bluntschli, H. J., and Goetz, R. H., *South African M. J.*, 1947, **21**, 382.

6 Freis, E. D., Stanton, J. R., and Wilkins, R. W., *Am. J. M. Sc.*, 1948, **216**, 163.

7 Rothlin, E., *Bull. schweiz. Akad. d. med. Wissensch.*, 1946-1947, **2**, 249.

8 Orth, O. S., Capps, R. A., and Suckle, H. M., *Fed. Proc.*, 1947, **6**, 361.

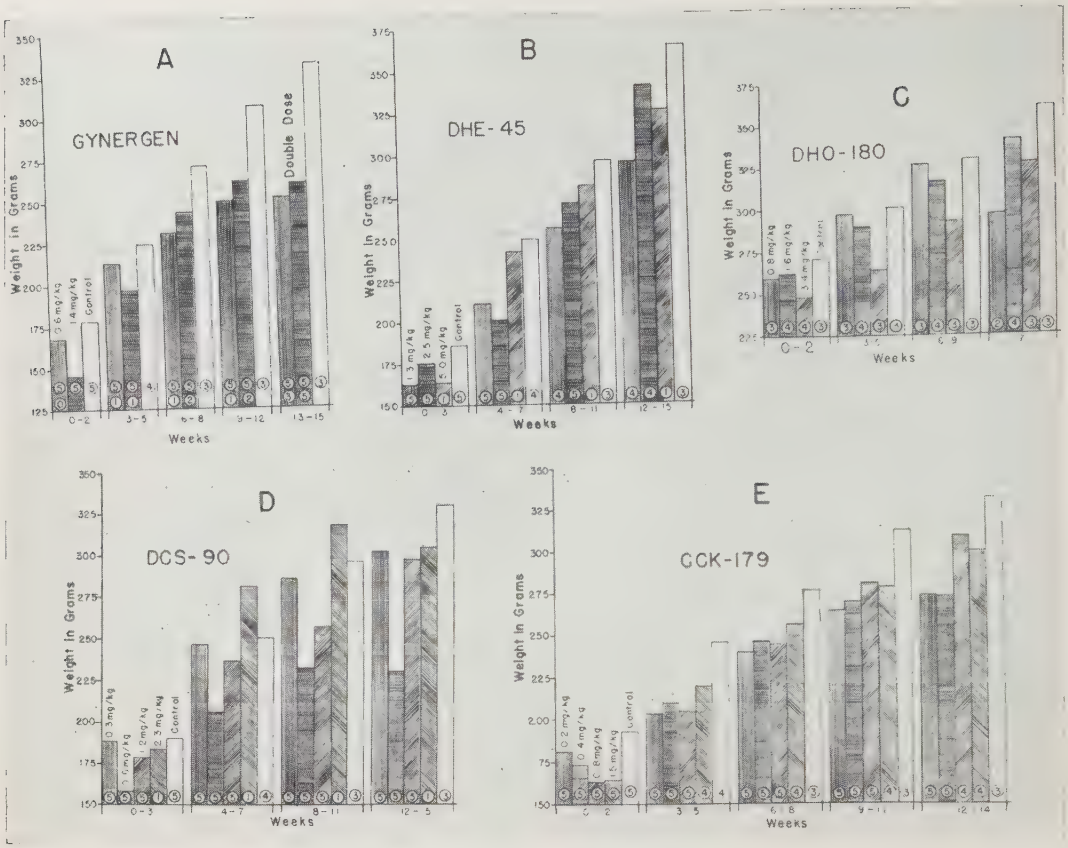


FIG. 1.

Effects of ergot products on growth rates, survival and production of gangrenous tails in rats.

A. Upper circle: Number of rats surviving the period. Lower circle: Number of rats with gangrenous tails at end of the period.

B to E, inclusive. Number encircled: Number of rats surviving the period.

tered by daily subcutaneous injection produced gangrene in about 4 weeks in about 20% of the rats. This is about 200 times the therapeutic dose in man.

The results of several weeks' weighings were averaged for simplicity in graphing. It was felt that this procedure was justified since the rate of growth of control rats was essentially linear. The results are shown in the figure.

*Results.* Ergotamine tartrate (Fig. 1, A) caused a decrease in the rate of growth in the doses employed. With doses as low as 0.6 mg/kg, one rat (20% of the rats) developed a gangrenous tail in 2 weeks. This dose was near the minimum for production of gangrene since doubling the dose caused an increase in the number of rats with gangrenous tails within 2 days. Survival was equal to or better than controls.

Dihydroergotamine (DHE 45), dihydroergocornine (DHO 180), dihydroergocristine (DCS 90), and a mixture of dihydroergocornine, dihydroergocristine and dihydroergokryptine (CCK 179) fail to produce a consistent inhibition of growth. These materials did not produce gangrene, and the survival of rats was equal to or better than controls.

*Summary.* The effect of several dihydrogenated derivatives of ergot were studied on rate of growth, production of gangrene and survival, as compared with ergotamine tartrate. None of these materials inhibited growth consistently; none inhibited growth as much as ergotamine tartrate. None of the dihydro-

genated derivatives caused gangrene; in contrast, ergotamine produced gangrene in 80% of the rats. The survival of rats receiving ergot alkaloids or dihydrogenated ergot derivatives was equal to or better than that of the controls.

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## Influence of Malononitrile upon Poliomyelitis in Mice.* (17316)

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Recent investigations by histophysical[1] and histochemical[2,3] methods identified the basophilic component of the Nissl bodies in the nerve cells as ribonucleic acid. Chromatolysis of the Nissl substance in the late preparalytic period is the earliest cytologic change in monkeys infected with poliomyelitis virus.[4] While chromatolysis is a reversible process, it may lead to complete cellular necrosis. Recovery is characterized histologically by the reappearance of the Nissl substance in the surviving nerve cells. Malononitrile $CH_2$ $(CN)_2$ increases the ribonucleic acid content, *i.e.*, the Nissl substance of the nerve cells but not of the liver and pancreas.[5] This prompted us to investigate the influence of malononitrile upon experimental poliomyelitis in mice.

*Materials and method.* Young, virus free mice, of the average weight of 12 to 15 g,† were infected with the Lansing strain of the poliomyelitis virus. The M.L.D. of this strain showed fluctuation during storage but was easily increased by successive mouse passages. The paralytic period, *i.e.*, the time between the appearance of the paralysis and death of the animal, was short, between a few minutes and 4 hours, even if low concentrations of the virus were used. The virus was inoculated intracerebrally, using 0.03 ml of a 10% mouse brain emulsion with an M.L.D. of $1.3 \times 10^{-3}$, $2.7 \times 10^{-5}$ and $3.2 \times 10^{-7}$, respectively. Malononitrile‡ was administered intraperitoneally, using a 0.5 g per L. solution sterilized by passage through a Seitz filter. Since malononitrile is toxic,[6] its effect on normal mice was investigated.

Two types of experiments were set up. In one, the survival rate of the infected mice treated daily with malononitrile before the appearance of paralysis was compared with that of untreated controls. In the second, mice were treated with malononitrile after the onset of the paralysis and the survival time was compared with that in untreated paralyzed mice. Because of the rapid downhill course of the disease in paralyzed animals, relatively few mice could receive treatment after the onset of paralysis. Thus many animals were lost. The survival time of mice was established in such manner that the hour at which the animal was last seen alive was considered as the last hour of its life.

After the death of the animals, brain, spinal cord, liver, heart and kidneys were fixed in Carnoy's or Bouin's fluid. In addition to

* Supported by grants from the Schering Research Fund and the Dr. Leonard H. and Louis Weissman Research Foundation.

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2 Brachet, J., *Enzymologia*, 1941, **10**, 87 and 96.

3 Gersh, I., and Bodian, D., *J. Cell. Comp. Physiol.*, 1943, **21**, 253.

4 Bodian, D., *Bull. Johns Hopkins Hosp.*, 1948, **83**, 1.

5 Hyden, H., and Hartelius, H., *Acta Psychiatr. et Neurol.*, Suppl., 1948, **48**, 1.

† Purchased from the Carworth Farms, N. Y.

‡ Received from the Schering Corporation, Bloomfield, N. J.

6 Heymans, J. F., and Masoin, P., *Arch. f. Internat. Pharmacodynam. a. Therap.*, 1897, **3**, 77.

TABLE I.
Effect of Malononitrile When Given in Preparalytic Phase.

| Virus M.L.D. | No. of mice | Malononitrile given | Avg incubation time, days | Mortality, % |
|---|---|---|---|---|
| $1.3 \times 10^{-3}$ | 24 | + | 37.1 ± 13.2 | 41.7 |
| | 24 | 0 | 10.2 ± 2.8 | 100 |
| $3.2 \times 10^{-7}$ | 84 | + | 5.3 ± 1.2 | 97.6 |
| | 84 | 0 | 4.4 ± 1.3 | 100 |

TABLE II.
Effect of Malononitrile Given in the Paralytic Period.

| Virus M.L.D. | No. of mice used | Malononitrile given | Avg survival time after onset of paralysis in hr |
|---|---|---|---|
| $2.7 \times 10^{-5}$ | 14 | + | 43.5 ± 8.7 |
| | 24 | 0 | 1.3 ± 0.4 |
| $3.2 \times 10^{-7}$ | 22 | + | 31.8 ± 6.1 |
| | 24 | 0 | 0.8 ± 0.2 |

hematoxylin-eosin, the sections were stained with gallocyanin and thionine (Windle's modification[7]).

*Results.* The intraperitoneal administration of 6 mg malononitrile per kg body weight to normal mice produced within a few minutes rapid respiration, convulsions and death. Three mg per kg body weight was well tolerated, although a general depression following an excitation state lasted frequently for many hours.

All 24 mice which received the Lansing virus of M.L.D. 1.3 x $10^{-3}$ died after an incubation period of 10.2 ± 2.8 days. Of 24 mice which received the same amount of the virus and 3 mg malononitrile per kg body weight the day after the infection, and, starting 6 days later, the same amount of the drug daily for 10 consecutive days, only 10, or 41.7%, died within 3 months. The average incubation time of the animals which died was 37.1 ± 13.2 days (Table I).

Of 84 mice injected with a high M.L.D. (3.2 x $10^{-7}$) all died. Of 84 animals infected with the same amount of the virus and treated with malononitrile, starting the day after infection, 82 died after an incubation time of 5.3 ± 1.2 days, which does not differ significantly from that of untreated animals (*i.e.*, 4.4 ± 1.3 days). Only 2 of the mice which received malononitrile survived in this group. The period between paralysis and death in the treated animals, however, was 17.8 ± 1.2 hours, as contrased with 0.8 ± 0.2 hour in untreated mice.

Of 38 mice infected with Lansing virus of M.L.D. 2.7 x $10^{-5}$, 24 served as controls for the determination of the survival time after paralysis developed, while 14 mice received 3 mg malononitrile per kg body weight after paralysis appeared. The average survival time of the treated animals was 43.5 ± 8.7 hours, while untreated animals lived only 1.3 ±0.4 hours after the onset of paralysis (Table II). Only 3 of the treated animals lived for less than 4 hours, while 2 survived for as long as 14 days. They showed complete recovery but died suddenly when the drug was withdrawn. In some of the other treated animals temporary partial or total disappearance of the paralysis was observed.

Of the 84 mice which received the virus in M.L.D. 3.2 x $10^{-7}$ concentration, 22 were treated with malononitrile after paralysis developed. Their survival time was 31.8 ± 6.1 hours, as compared with 0.8 ± 0.2 hour in 24 control animals. About one-third of the treated mice showed partial or complete recovery from the paralysis but died later.

The treated animals which survived for three months did not show the usual histological involvement of the central nervous sys-

[7] Windle, W. F., Rhines, R., and Rankin, J., *Stain Technol.*, 1943, **18**, 77.

tem,[8] while those which died despite the treatment showed similar pathologic changes as the untreated mice except that the cytoplasmic basophilic substance of the nerve cells appeared better preserved.

*Discussion.* The described experiments indicate that malononitrile has a certain value in the treatment of mouse poliomyelitis. If lower concentrations of the virus were used, treatment in the preparalytic stage prevented death in more than one-half of the animals and prolonged the incubation period nearly threefold. While death following huge doses of the virus could not be prevented by the drug, the life span between paralysis and death was prolonged also in these cases.

When malononitrile was given after the appearance of paralysis, in about one-third of the animals significant regression of the clinical symptoms was observed. Complete recovery was observed in solitary cases but the animals died after cessation of the treatment.

There are various ways in which a chemical compound may act against the poliomyelitis virus. It may have a prophylactic effect either due to the action upon the virus before it reaches the nervous system, or it may render the nerve cells unsuitable for the multiplication of the virus. The arrangement of these experiments precludes the possibility of a direct inactivation of the virus before it reached the nervous system. Malononitrile, however, may render the intracellular environment unsuitable for continued development of the virus by its protective effect upon the nucleic acids. It may also help to restore the damaged function by increasing the ribonucleic acid content of the attacked cells. While a direct action upon the virus within the cell is not probable, the possibility that this compound acts by interfering with an enzyme system of the host, essential for the multiplication of the virus, has to be considered.

*Summary.* Malononitrile treatment preserved the life of more than one-half of the mice infected with a low concentration of the Lansing type poliomyelitis virus, but only prolonged the life span of mice infected with large doses of the virus. Mice treated with malononitrile after the onset of the paralysis showed markedly prolonged survival time. In about one-third of the animals partial or total recovery of the paralytic symptoms was observed; the mice, however, died after cessation of the treatment.

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[8] Lillie, D. R., and Armstrong, C., *Publ. Health Rep.*, 1940, **55**, 718.

## Change of Muscular Excitability by Eserine, Acetone and Methyl-alcohol. (17317)

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The potentiation of acetylcholine by eserine, acetone, and methyl alcohol being known, we are interested in finding out their influence on the electrical excitability of the muscle.

The rheobase and chronaxie were determined with condenser discharge. The rectus muscle of the toad was placed in a glass muscle chamber, moistened with Ringer's solution, and observed under dissecting microscope. After the control reading had been taken, the rectus muscle was immersed in a bath of 8 cc of Ringer's solution, bubbled with air, and treated with 10 $\gamma$ eserine for 30 minutes, or 0.05 cc acetone or methyl-alcohol for 3 minutes, and, in addition, Ringer's solution was alone used without adding any reagent for blank control. Following the treatment, both were redetermined under conditions as before.

TABLE I.
Excitability of Rectus Muscle Increased by Eserine, Acetone, and Methyl Alcohol.

| | | Rheobase | | | | |
|---|---|---|---|---|---|---|
| | | Before treatment | | After treatment | | |
| Treatment | No. of observations | Range, v. | Avg, v. | Range, v. | Avg, v. | Difference, v. |
| Eserine | 10 | .58- .71 | .669 ± .014 | .34- .58 | .485 ± .024 | .184 ± .028 |
| Acetone | 10 | .65- .78 | .717 ± .014 | .46- .66 | .583 ± .020 | .134 ± .024 |
| Methyl-alcohol | 10 | .65- .77 | .705 ± .012 | .44- .66 | .569 ± .019 | .136 ± .022 |
| Ringer's solution (Blank control) | 10 | .92-1.34 | 1.106 ± .041 | .90-1.33 | 1.085 ± .044 | .021 ± .060 |

| | | Chronaxie | | | | |
|---|---|---|---|---|---|---|
| | | Before treatment | | After treatment | | |
| Treatment | No. of observations | Range, msec. | Avg, msec. | Range, msec. | Avg, msec. | Difference, msec. |
| Eserine | 10 | 1.30-7.46 | 3.649 ± .563 | 1.11- 8.14 | 4.007 ± .782 | .358 ± .964 |
| Acetone | 10 | .40-8.55 | 3.086 ± .710 | 1.54-11.0 | 3.859 ± .830 | .773 ± 1.092 |
| Methyl-alcohol | 10 | .77-6.22 | 4.104 ± .535 | 1.72- 6.75 | 4.810 ± .532 | .706 ± .754 |
| Ringer's solution (Blank control) | 10 | .87-7.96 | 4.064 ± .788 | 1.91- 9.64 | 5.308 ± .877 | 1.244 ± 1.187 |

It was found that rheobase was uniformly decreased by all these agents (Table I), indicating that the excitability of the muscle was definitely increased according to Chao.[1] Similar treatment with Ringer's solution gave no significant change.

[1] Chao, I., *The Science Reports of National Tsing Hua Univ. Series B*, **2**, 183.

The chronaxie showed insignificant change, and we agree with Chao[1] that it is not a valid measure of excitability in such a case.

*Summary.* With the threshold intensity as a criterion, the excitability of the rectus muscle is definitely increased by eserine, acetone and methyl-alcohol.

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## Detection of Acrolein by Qualitative Immunochemical Analysis. (17318)

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In a previous report[1] it was shown that acrolein can produce a condition in experimental animals similar in its clinical and pathologic manifestations to that accepted as "shock". The problem of the detection of acrolein in blood or tissue by some specific method next presented itself. Acrolein is found to be a chemically active and highly unstable compound that does not retain its characteristics for any length of time. These two properties alone would make it difficult to detect by the usual qualitative methods of organic analysis, especially when acrolein is present in small quantities. Secondly, as will be shown, there is experimental evidence indicating that acrolein combines directly with protein, and no longer exists in a free state. It thus becomes obvious that the usual qualitative chemical methods of detection cannot be used.

[1] Kamen, G. F., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 363.

Another possible method for the detection was suggested by a postulated chemical com-

bination of acrolein with protein. If such a reaction takes place in the body, then it should follow that the configuration and chemical properties of the native protein molecule would be changed. Theoretically, protein molecules changed in this manner behave as substances foreign to the body and may act in the capacity of an antigen and cause antibody formation. Applying this principle to the pathologic physiology of burns and burn shock, one might assume that any acrolein that may be produced as a result of a burn probably combines with body protein locally or systemically. In the combined state it theoretically might be detected. Animal experimental studies were undertaken to demonstrate, if possible, by indirect evidence chemical changes in protein molecules treated with acrolein, in order to test the validity of these considerations.

*Laboratory procedure.* Freshly distilled acrolein was added to normal human and rabbit sera, in sufficient quantity so that the mixtures contained 0.5 and 1.0% of acrolein by volume. These mixtures were immediately diluted 1:5 with physiological salt solution. Sheep serum was prepared in the same manner to contain 1.5% of acrolein. The flasks were stoppered with cotton; all were then placed in a constant temperature water bath at 37°C, some for 4 days and others for 10 days to permit any excess acrolein to volatilize. At the end of this time, the contents were transferred to diaphragm rubber-stoppered bottles that were kept at refrigerator temperature (5°C). The acroleinized serum incubated at 37°C for 10 days undergoes further changes after approximately one month and will give nonspecific precipitin reactions when mixed with normal horse, rabbit, sheep or human serum. The acroleinized serum incubated at 37°C for 4 days and kept for as long as nine months was found to be free from nonspecific reactions. The microprecipitin test was used; the reactions were read within 5 minutes at room temperature. For the possible demonstration of cross reactions with formolized serum, normal human and rabbit sera were each prepared to contain 0.8% formalin gas and placed in a constant temperature water bath at 37°C for 10 days with cotton stoppers to allow any excess formalin to volatilize. On the tenth day the formalized serum was removed and diluted 1:5 with physiologic saline solution and transferred to diaphragm rubber-stopped bottles and stored at room temperature.

*Immunization.* Male rabbits, approximately 7 lb in weight and 5 to 6 months of age, were used. All injections were given intravenously. Acroleinized human serum incubated for 10 days was injected daily for an average of 6 days. After this a rest period of a week was allowed. The injections were repeated in this manner for a series of 4 courses of injections. The daily amount given in the first series was 0.5 ml, 1.0 ml, in the second, 2.0 ml, in the third and the final series of injections 3.0 ml. Acroleinized rabbit serum incubated for 10 days was injected in a like manner except, the first course of injections was 1.0 ml daily of 1:10 dilution; in the next 5 courses of injections a 1:5 dilution was used. The amount injected daily in the second series of injections was 1.0 ml, third 2.0 ml, fourth 3.0 ml, fifth 3.0 ml. In the sixth and final series 5.0 ml were injected every other day of the 6 day period.

Eight days after the completion of the series of injections, 3.0 ml of blood were collected from the ear vein of each of the animals receiving acroleinized serum incubated for 10 days and the antibody titer determined. Later the same day, 40.0 ml of blood were removed from the heart of each animal. To determine if bleeding would influence the antibody titer, an additional series of injections of acroleinized serum after the third series was then given as outlined above. Antibody titer increases were only slight.

Acroleinized human or rabbit serum incubated for 4 days was injected in daily single doses of 1.0 ml initially, 2.0 ml for the second, 3.0 ml for the third, and 3.0 ml for the fourth and final series of injections. Ten days following the last injection each animal was bled from the heart of approximately 50 ml.

Formalized human or rabbit serum was injected daily for 6 days and then a rest period of about a week was allowed. This was repeated for 5 courses of injections. The amount given initially at each injection was 1.0 ml,

TABLE I.

| Antiserum | Test antigen | Antigen dilution 1:5 | 1:25 | 1:125 | 1:625 | 1:3125 | Titers |
|---|---|---|---|---|---|---|---|
| Anti-acroleinized human serum | Acroleinized human serum | ++++ | ++++ | ++++ | + | — | 1:125 |
| | Normal human serum | ++++ | ++++ | ++++ | ++ | — | 1:625 |
| | Acroleinized rabbit serum | ++++ | ++++ | ++ | — | — | 1:125 |
| | Normal rabbit serum | — | — | — | — | — | 0 |
| | Acroleinized sheep serum | ++++ | ++++ | +++ | + | — | 1:125 |
| | Normal sheep serum | — | — | — | — | — | 0 |
| | Acrolein | — | — | — | — | — | 0 |
| Anti-acroleinized rabbit serum* | | — | | | | | |

* No precipitins demonstrated against any of the test antigens including acroleinized rabbit serum.

1.0 ml for the second and third, 2.0 ml for the fourth and 3.0 ml for the final series of injections. A preliminary bleeding one week after the third series of injections showed, in those rabbits receiving human serum, precipitins that reacted against normal human serum and formalized human serum, but not against normal or formalized rabbit serum. No precipitins could be demonstrated in the blood of those rabbits that received rabbit serum. The formalin concentration was then adjusted from 0.8% to 2.0% (formalin gas) and incubated at 37°C for 24 hours in sealed flasks. Two additional series of injections were then given and the rabbits bled from the heart 9 days after the last injection. The precipitin reactions were the same as before.

*Results.* Results are summarized in the table. While the rabbits injected with acroleinized rabbit serum did not respond, those injected with acroleinized human serum did produce precipitins. These precipitins reacted not only with acroleinized human serum but also with acroleinized rabbit and acroleinized sheep serum. As expected, they also reacted with normal human serum due to the formation of precipitins for normal human serum protein. That the cross reactions with acroleinized rabbit and sheep sera were due to an antibody directed against acrolein in a combined form with protein itself is indicated by the observation that the antiserum did not react with untreated rabbit or sheep serum and also did not react with pure acrolein.

*Discussion.* The molecular configuration of rabbit serum protein is altered by acrolein but not sufficiently to act in the capacity of an antigen and cause antibody formation when injected into rabbits. Apparently the basic molecular structure of the protein characteristic of the human and rabbit species is not altered by acrolein. This is shown by the failure to form antibodies when rabbits are injected with acroleinized rabbit serum, and also by the response of the rabbits injected with acroleinized human serum by producing antibodies that reacted not only against acroleinized human serum but with also normal human serum proteins. Slight changes in the configuration of the protein molecules, however, did occur. These changes remained the same regardless of the basic molecular structure of the protein as evidenced by cross reactions of antiacroleinized human serum with acroleinized serum of other species. That the antibody was directed against the acrolein in a combined form with protein itself is indicated by the observation that the antiserum did not react with untreated rabbit or sheep serum and also did not react with pure acrolein. It would seem, therefore, that foreign protein conjugates of acrolein are necessary to produce circulating antibodies that can be detected. From the chemical nature of acrolein and protein, it may be that this linkage takes place through indole nitrogen and amino

groups.

*Conclusion.* 1. These studies indicate that acrolein combines directly with some portion of the protein molecule to produce a conjugate that can be employed in the specific detection of acrolein by qualitative immunochemical methods.

2. The basic molecular pattern of human and rabbit serum protein is not changed when combined with acrolein at 37°C for 4 days. The addition of molecules of acrolein, however, does change the configuration of the protein molecule without loss of species characteristics. This is shown by the failure to form antibodies when rabbits are injected with acroleinized rabbit serum, and also by the response of the rabbits injected with acroleinized human serum by producing antibodies that reacted not only against acroleinized human serum but normal human serum as well. These changes in the molecular configuration were specific for acrolein regardless of the basic molecular pattern used, as evidenced by cross reactions of antiacroleinized human serum with acroleinized serum of other species.

3. Foreign protein conjugates of acrolein are necessary to produce circulating antibodies that can be detected in a test tube.

Studies are now in progress on the detection of acrolein in the blood and tissues of individuals who have suffered burns.

I wish to acknowledge the helpful advice and cooperation of Dr. Alexander S. Wiener and his laboratory personnel.

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## Inhibitory Effect of Nitrogen Mustard (Bis Beta-Chloroethyl Amine) on Lesions of Experimental Serum Hypersensitiveness.* (17319)

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Experimental serum hypersensitiveness has been studied intensively because it may serve as a useful model in promoting understanding of certain diffuse vascular diseases appearing in man. The earlier investigations of experimental hypersensitiveness were concerned primarily with morphologic characteristics of the lesions and their similarity to those of the human diseases. More recent investigations, concerned with pathogenesis, have dealt with the relationship of immunologic and vascular changes.[1-4] The information obtained has led to the general conclusion that the development of humoral antibody and cutaneous hypersensitiveness is apparently related to the development of the vascular lesions. The role of immunological factors has been further investigated by determining the incidence of vascular lesions in serum-injected animals treated with drugs which may 1) prevent antigen-antibody combination or the vascular response to such combination or 2) inhibit antibody formation. Salicylates and dicumarol inhibit antigen-antibody combination *in vitro*,[5,6] but as yet there is no

* This investigation was supported by a grant from the U. S. Public Health Service.

1 Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, **85**, 571.

2 Fox, R. A., and Jones, L. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 294.

3 Hopps, H. C., and Wissler, R. W., *J. Lab. Clin. Med.*, 1946, **31**, 939.

4 Sullivan, C. J., Parker, T. W., and Hibbert, R. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 508.

5 Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, **77**, 173.

6 Forman, C., Seifter, J., and Ehrich, W. H., *J. Allergy*, in press.

evidence that this effect occurs *in vivo*. That salicylates suppress the vascular lesions of serum hypersensitiveness has been reported;[4,6,7] the mechanism of this action has not been clarified. Since some of the cellular responses to antigen-antibody combination may be related to histamine release, antihistaminic drugs have been used in attempts to suppress the lesions of experimental serum hypersensitiveness. While such an effect has been reported,[8,9] our own efforts to prevent the vascular lesions with benadryl and neohetramine have been unsuccessful.[10]

Experiments designed to suppress antibody formation were undertaken in an attempt to establish the pathogenetic importance of antibody. Recent evidence of the importance of the lymphatic system in production of antibody suggested the use of lymphocytotoxic agents for this purpose, particularly since Hektoen and Corper[11] had inhibited antibody formation with sulfur mustards and recent experimental studies[12,13] have demonstrated similar effects by the nitrogen mustards. Furthermore, nitrogen mustard has been found capable of reducing immunity in chicken malaria.[14]

In the present study we have found that the nitrogen mustard, bis betachloroethyl amine ($HN_2$) suppresses antibody formation, the development of cutaneous hypersensitiveness, and the incidence and severity of vascular lesions in rabbits receiving one intravenous injection of 10 ml of horse serum per kg. The results obtained suggest that the appearance of vascular lesions bears a direct quantitative relationship to the antibody response of the experimental animal.

*Experimental.* Twenty rabbits were studied in this investigation. Ten rabbits were treated with $HN_2$ and received intravenous serum, while an additional 10 rabbits were given serum alone.

Intravenous injections of 0.5 mg of $HN_2$/kg were made at 3-4 day intervals for 3 doses prior to intravenous injection of horse serum. Four additional injections at the same intervals during the subsequent 2 week period preceded sacrifice. Leucocytes were enumrated before administration of mustards, after the 5th injection (1 week after serum) and upon completion of the experiment. Qualitative serologic tests were performed by a tube precipitation procedure while quantitative antibody nitrogen (N) determinations were made colorimetrically by the technic of Heidelberger and MacPherson.[15] Skin tests, using 5 dilutions of horse serum, were graded according to the presence of hemorrhage and the area of edema and erythema observed 24 and 48 hours after intradermal injection of 0.2 ml.

Ten animals were observed in each of 2 experiments. In the first experiment antigen persisted in the circulation for the duration of observation in all but one of the animals. The antibody response is graphically represented in Fig. 1, in which the upper 5 curves represent antibody titers of serum controls while the lower 5 are for the $HN_2$ treated group. It is quite evident that antibody was delayed in appearance and much reduced in amount in the $HN_2$ treated group. This finding is even more evident in the quantitative studies of the terminal sera of all animals as indicated in Fig. 2.

The figures on each curve represent the rabbit number and, in parentheses, the calculated total antibody N content per ml of undiluted serum. Curves for the $HN_2$ treated group are flat and the total antibody N content does not exceed .036 mg per ml, while the content of the controls varies from a minimum of .060 to .152 mg per ml.

[7] Smull, K., Wissler, R. W., and Watson, J. M., *J. Lab. Clin. Med.*, 1948, **33**, 939.

[8] Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. Clin. Med.*, 1947, **32**, 379.

[9] Kyser, F. A., *Quart. Bull. Northwestern Univ. Med. School*, 1948, **22**, 256.

[10] Dammin, G. J., Bukantz, S. C., and Alexander, H. L., *J.A.M.A.*, 1949, **139**, 348.

[11] Hektoen, L., and Corper, H. J., *J. Inf. Dis.*, 1921, **28**, 279.

[12] Phillips, F. S., Hopkins, F. H., and Freeman, M. L. H., *J. Immunol.*, 1947, **55**, 289.

[13] Spurr, C. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 259.

[14] Taliaferro, W. H., and Taliaferro, L. G., *J. Inf. Dis.*, 1948, **82**, 5.

[15] Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, **97**, 405; **98**, 63.

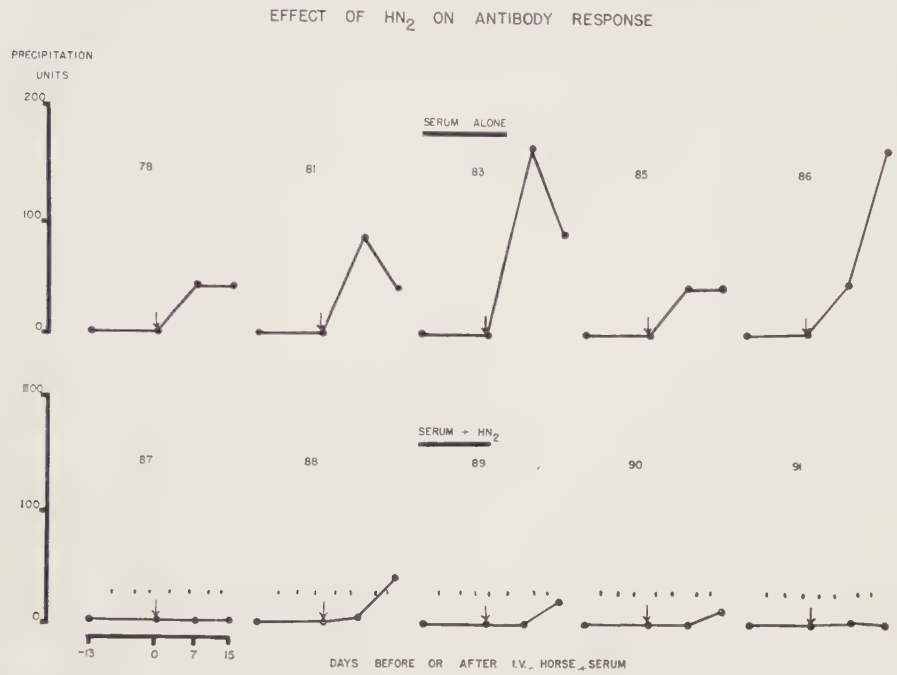


FIG. 1.
Experiment I (see text).

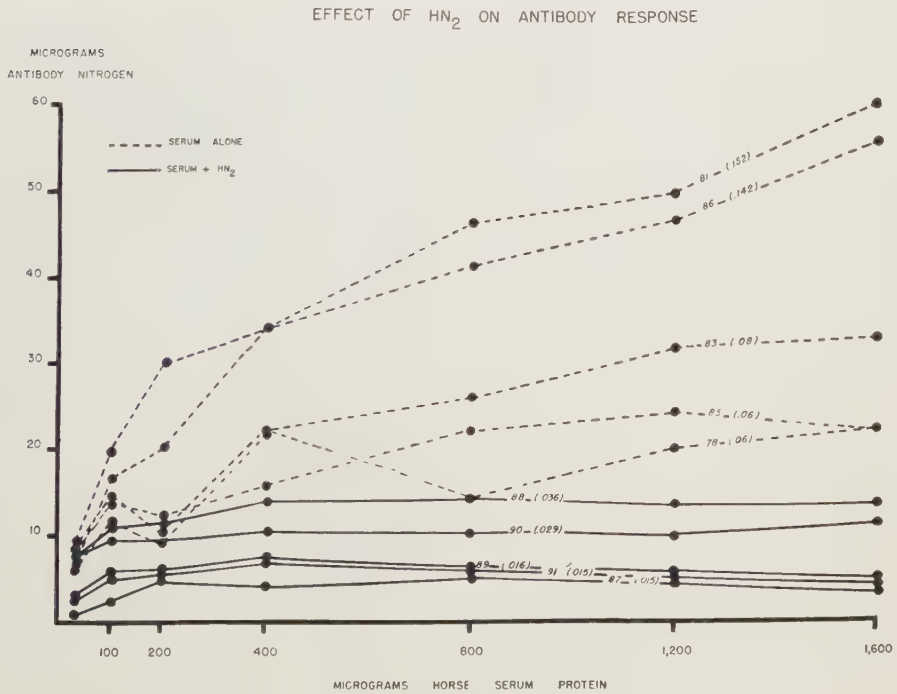


FIG. 2.
Experiment I (see text).

TABLE I.
Exp. I. Effect of $HN_2$ on Response to I.V. Horse Serum.

| Group | Rabbit No. | Antibody† N, mg/ml | W.B.C. Before* | W.B.C. After* | Arthus‡ 1:1§ | 1:5 | 1:10 | 1:20 | 1:40 | Vascular lesions |
|---|---|---|---|---|---|---|---|---|---|---|
| $HN_2$ treated | 87 | .015 | 12,700 | 2,700 | ++ | + | + | + | 0 | 0 |
| | 88 | .036 | 9,050 | 3,200 | ++ | + | + | + | 0 | 0 |
| | 89 | .016 | 10,250 | 1,850 | ++ | ++ | + | + | 0 | 0 |
| | 90 | .029 | 10,200 | 1,400 | ++++ | +++ | +++ | ++ | ++ | 0 |
| | 91 | .015 | 9,350 | 2,350 | +++ | ++ | + | + | 0 | 0 |
| Control | 78 | .058 | | | ++++ | ++++ | +++ | +++ | ++ | 0 |
| | 81 | .15+ | | | ++++ | ++++ | +++ | +++ | +++ | + |
| | 83 | .08 | | | ++++ | ++++ | +++ | +++ | + | + |
| | 85 | .06 | | | ++++ | ++++ | ++ | ++ | 0 | + |
| | 86 | .14+ | | | ++++ | ++++ | ++ | ++ | ++ | + |

* Completion of $HN_2$ Rx.
† Terminal serum.
‡ 13-14 days after serum.
§ Dilution of horse serum injected intradermally.

Table I summarizes the significant findings of Experiment I. The quantities of antibody N again illustrate the suppressive effect of $HN_2$ therapy. A significant leucopenia (of both granulocytes and lymphocytes) developed in all $HN_2$ treated animals. In general, Arthus reactions in the $HN_2$ treated group were less intense than those in the control group and therefore paralleled the antibody levels. Skin reactivity did not, however, correlate perfectly with antibody level as indicated by rabbit No. 90, which with 0.029 mg antibody N/ml yielded reactions equal in intensity to No. 86 with .14 mg/ml. None of the $HN_2$ treated rabbits developed vascular lesions while arterial and/or endocardial lesions of characteristic type were encountered in 4 of the 5 serum control animals. The type of arterial lesion observed is illustrated in Fig. 3.

The results of the quantitative antibody determinations on the terminal sera of Experiment II are summarized in Table II and Fig. 4. Three of the $HN_2$ treated group failed to develop any antibody despite the fact that antibody N production by the controls was considerably greater than in the first experiment. One of the 2 remaining $HN_2$ treated rabbits (5) in this experiment, however, produced amounts of antibody greater than 4 of the 5 controls and the other (M-4) more than that produced by $HN_2$ treated rabbits of the first experiment. Among the $HN_2$ treated rabbits, 3 and M-1, which had developed evident leucopenia under treatment, developed no antibody, yielded completely negative Arthus reactions and failed to develop vascular lesions. Rabbit M-5 which died 8 days after serum injection developed evident vascular lesions, for which we have no satisfactory explanation. Rabbits 5 and M-4 showed lesser degrees of leucopenia and developed considerably more antibody than was common to the $HN_2$ treated groups; they also exhibited evident positive Arthus reac-

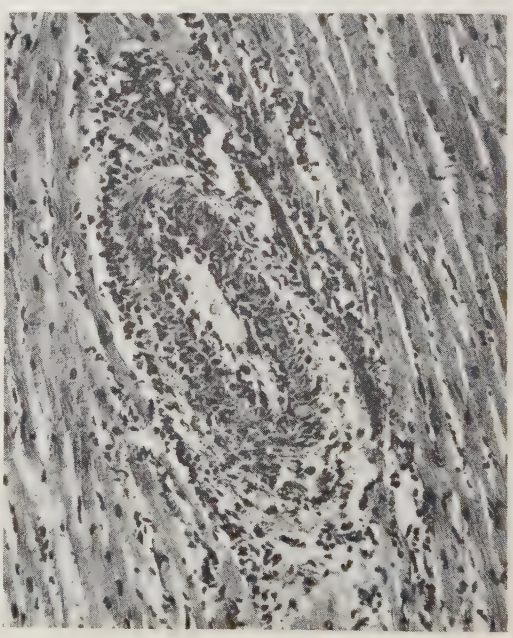

FIG. 3.
A myocardial artery (from a serum control animal) exhibiting marked acute panarteritis, and well-defined medial necrosis.

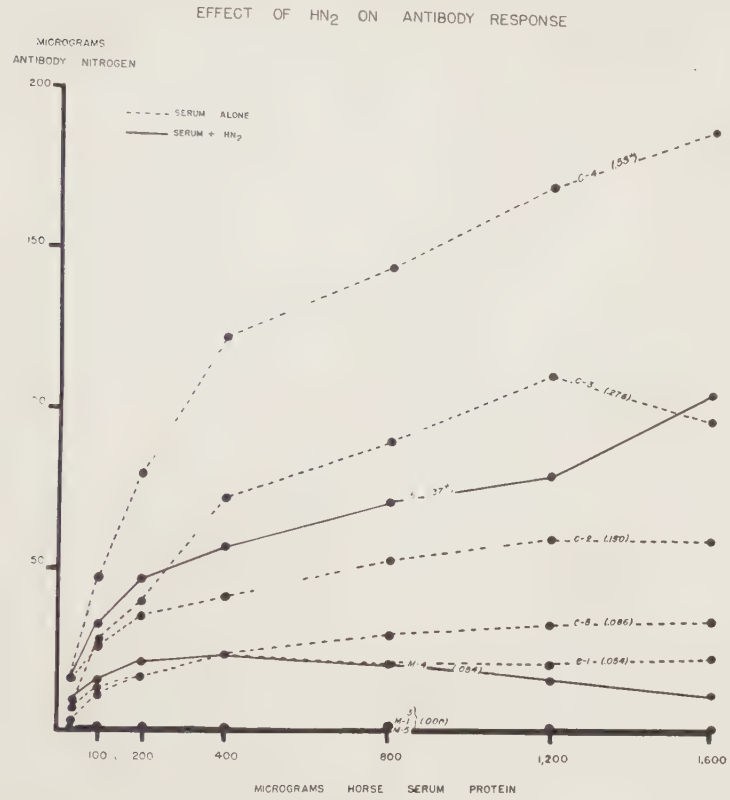


FIG. 4.
Experiment II (see text).

tions and slight vascular lesions. It was our impression that these rabbits, reacting almost exactly as untreated rabbits, had been relatively unresponsive to $HN_2$.

Control rabbits in this experiment exhibited a fairly wide variation in total antibody N and no evidence of leucopenia at the completion of the experiment. The severity of the vascular lesions developed by 4 of the controls was directly correlated with antibody level, the mildest lesions occurring in rabbit C-5 and the most severe in C-4. The correlation between the intensity of the Arthus reactions and the level of antibody was again imperfect, there being considerable variability in skin responsiveness.

In general, the 2 experiments reveal that the intensity of the vascular lesions correlated fairly directly with the levels of antibody N in the terminal serum samples, whether the animals had been treated with $HN_2$ or not.

The dividing line between the appearance of vascular lesions and their absence is at approximately .06 mg antibody N/ml. All rabbits with greater amounts of antibody developed vascular lesions, including one $HN_2$ treated rabbit (5) of experiment II. All animals with less than .05 mg antibody N/ml failed to develop vascular lesions, with the exception of the one to which reference has already been made (M-5).

*Discussion.* The vascular lesions of experimental serum hypersensitiveness occur in animals treated in a manner known to induce the anaphylactic and Arthus type of hypersensitiveness. The importance of antibody in the latter 2 hypersensitive states has been repeatedly demonstrated[16-19] and it would, therefore, seem logical to conclude *a priori*

16 Opie, E. L., *J. Immunol.*, 1924, **9**, 231.

17 Culbertson, J. T., *J. Immunol.*, 1935, **29**, 29.

18 Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1941, **40**, 127.

19 Kabat, E. A., *Am. J. Med.*, 1947, **3**, 535.

TABLE II.
Exp. II. Effect of $HN_2$ on Response to I.V. Horse Serum.

| Group | Rabbit No. | Antibody N, mg/ml | W.B.C. Before | W.B.C. After | Arthus 1:5 | Arthus 1:10 | Arthus 1:20 | Arthus 1:40 | Arthus 1:80 | Vascular lesions |
|---|---|---|---|---|---|---|---|---|---|---|
| $HN_2$ | M-1 | 0 | 8,600 | 1,200 | 0 | 0 | 0 | 0 | 0 | 0 |
| treated | M-4 | .054 | 11,950 | 3,600 | +++ | +++ | ++ | + | ∓ | ± |
| | 3 | 0 | 11,500 | 2,400 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 5 | .37+ | 9,000 | 4,500 | ++ | ++ | +± | +± | + | ± |
| | M-5* | 0 | 9,300 | 2,900 | | | Not done | | | + |
| Control | C-1 | .05 | — | 6,650 | ++ | ++ | ± | ± | 0 | 0 |
| | C-2 | .15 | — | 6,700 | ++ | ++ | +± | 0 | 0 | ± |
| | C-3 | .276 | — | 5,750 | ++++ | ++++ | +++ | +++ | + | + |
| | C-4 | .55+ | — | 10,000 | +++ | ++ | ++ | +± | + | + |
| | C-5 | .086 | — | 6,400 | ++ | + | + | ∓ | 0 | ± |

* Died 8 days after serum I.V.

that the development of vascular lesions by the serum-injected rabbit would also be related to the development of antibody. Nevertheless, several previous studies are not in agreement with this conclusion.[3,7]

In the present experiments, the nitrogen mustard, bis beta-chloroethyl amine induced leucopenia and a parallel suppression of both antibody formation and vascular lesions. While suggestive, these studies, however, are not interpreted as establishing the role of antiprotein antibody in the pathogenesis of the experimental vascular lesions. The nitrogen mustards are powerful inhibitors of a number of enzyme systems and of a variety of tissue metabolic functions; some of the systems affected may possibly play a role in the pathogenesis of the vascular lesions, independent of the antibody present. It would be desirable, in defining the role of antibody, to reproduce the lesions by passive transfer methods, to study the effects of x-rays, and to extend the present observations to include larger groups of animals before attempting to formulate recommendations regarding management of related human diseases.

*Summary.* Periodic intravenous injections of the nitrogen mustard, bis beta-chloroethyl amine, suppressed both antibody formation and the development of vascular lesions in rabbits injected intravenously with a massive dose of horse serum. The incidence of vascular lesions was directly correlated with the amount of antibody produced, since all rabbits with terminal amounts of antibody in excess of 0.06 mg nitrogen per ml exhibited vascular lesions. Cutaneous hypersensitiveness failed to develop in rabbits which did not produce detectable amounts of antibody.

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# Shrinking and Swelling after Alpha Irradiation of Various Parts of Large Erythrocytes.* (17320)

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It is well known that ionizing radiations produce hemolysis of red blood cells *in vitro*, and this seems to be associated with swelling.[1] Nearly all investigators have studied these phenomena with large populations of erythrocytes so that variation in behavior of the individual cells can be smoothed out to get reproducible data. As far as we know, only one previous worker has used a microbeam to irradiate portions of a single cell. Tchakhotine[2] irradiated egg cells with ultraviolet rays focused to a 5 micron "point" and described local damage of the membrane and underlying cytoplasm. We are aware of no similar studies with ionizing radiation.

In this study we have sacrificed the many advantages of using massive populations of erythrocytes in order to direct our attention to the microscopic changes that can be observed in individual cells before, during and after irradiation. This method has revealed a striking effect that seems to have escaped attention. Using erythrocytes of Amphiuma and of man, we have observed that between the time of irradiation and the well-known swelling and hemolysis there occurs a shrinkage of the cells. After total alpha irradiation of a cell the whole cell shrinks. After irradiation of a portion of a cell only the irradiated portion shrinks.

*Methods and materials.* Two methods of irradiation were used, total cell irradiation in which cells in tissue cultures on coverslips were exposed, and microbeam or partial cell irradiation in which only portions of cells were exposed. Two sources of alpha particles were used.† For total cell irradiation the source was polonium deposited on a palladium disc 4 mm in diameter. This was mounted in a brass holder which simulated a microscope objective and could be screwed into the nosepiece of the microscope. In some experiments irradiation was administered from above by selecting a suitable field and then revolving the nosepiece to bring the sourceholder over it.[3] In other experiments the source-holder was mounted in the microscope condenser-holder so that cells could be irradiated from below. In the latter case, the cells were first observed with the condenser in place, then irradiated by substituting the source for the condenser, and finally observed with the condenser back in place. Alternatively, the cells could be observed continuously before, during and after irradiation by using vertical illumination without the exchanges of source and condenser.

For microbeam or partial cell irradiation a smaller source was used. The polonium was deposited on the end of a palladium wire, 0.3 mm in diameter, which was then inserted into a steel sheath made from hypodermic needle. One end of the sheath was covered with an aluminum cap, the top of which was perforated with a small hole for the exit of alpha particles. This small source was mounted on the condenser-holder. In use, a preparation was focused on the microscope stage with vertical illumination. The body tube was then racked down 20 $\mu$, and the condenser-holder bearing the source racked up until the top of the aluminum cover with the

* This work was done under Contract N6ori-20 with the Office of Naval Research and with the cooperation of the United States Atomic Energy Commission.

1 Ting, T. P., and Zirkle, R. E., *J. Cell. and Comp. Physiol.*, 1940, **16**, 189.

2 Tchakhotine, S., *Compt. rend. soc. biol.*, 1935, **120**, 714.

† The sources were furnished by the Chemistry Division of Argonne National Laboratory. We are especially indebted to Dr. W. M. Manning and Mr. P. R. Fields for their courtesy and to Mrs. Sylvia Warshaw for her services in preparing the sources.

3 Zirkle, R. E., *J. Cell. and Comp. Physiol.*, 1932, **2**, 251.

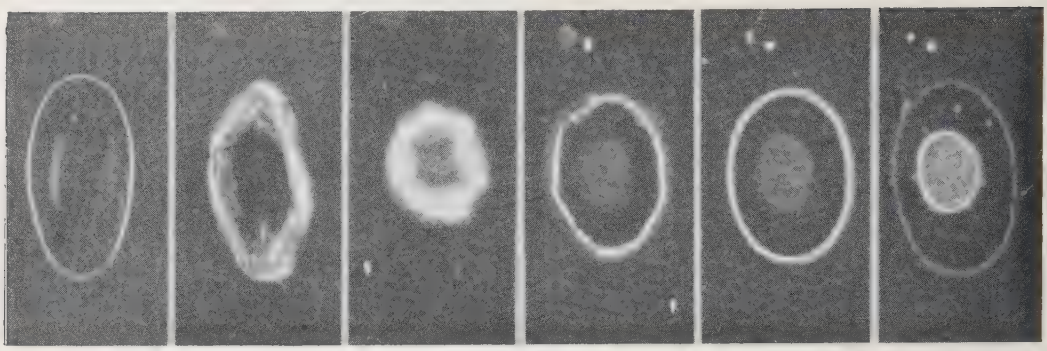

FIG. 1.
Photomicrographs of an Amphiuma erythrocyte showing normal cell, shrinkage, swelling, and hemolysis, after $5 \times 10^5$ rep of alpha radiation. Vertical illumination. Length of normal cell, 75 $\mu$.

hole came into focus. By this procedure the source was brought as close as feasible to the cells. The hole was centered with respect to ocular cross hairs. To make an exposure a piece of coverslip was withdrawn and reinserted as a shutter in the space between the aluminum cap of the sheath and the 4 $\mu$ mica slip at the bottom of the preparation.

The radiation consisted exclusively of polonium alpha particles (energy 5.3 Mev). Since these particles have a total range of only about 35 $\mu$ in tissue, the path from the source to the cells was made as short as possible to permit maximal penetration into the cells. The 4 mm source had an initial activity of 790 $\mu$c. The distance between source and cell was 490 $\mu$ and the dose rate was calculated to be about 132,000 rep per minute. Exposures lasted from one to 50 minutes. The microbeam source had an activity of 275 $\mu$c at the start of these experiments. The total number of particles reaching the field of irradiation was determined by direct count of those passing through the small holes used. In the case of the 21.6 $\mu$ hole, which was used most extensively, 12,500 particles emerged per minute. The area covered by the particles was determined directly. An Eastman NTA plate was substituted for a cell preparation and bombarded for one minute. A photograph of a stage micrometer was also made at the same time. Superimposing these two images gave the area of bombardment. Exposures ranged from one to 90 minutes; the average dose rate was 84,000 rep per minute. Time of exposure was corrected for radioactive decay of the sources.

Cell preparations were made as follows. After partial caudal amputation, a drop of Amphiuma blood was allowed to fall upon a coverslip and was covered immediately with a mica coverslip 3.5 to 4.5 $\mu$ thick (0.7 to 0.8 mg per $cm^2$). Blood from man was drawn by skin puncture. The preparations were sealed with vaseline. Cell changes were recorded both by fast-motion cine-photomicrography and by still photomicrography. Cytological details not visible by vertical illumination were studied with phase microscopy.

*Results of Total Cell Irradiation.* Groups of irradiated erythrocytes from Amphiuma or man generally show the same type of response with respect to hemolysis. A typical response is shown in Fig. 1. After irradiation the cells shrink, becoming very wrinkled. Later they swell until they reach or exceed their initial size, after which they hemolyze. Cells which shrink the earliest and to the greatest extent take the longest to swell and to hemolyze.

*Results of partial cell irradiation.* Individual Amphiuma erythrocytes, after irradiation as described above, shrink in the irradiated portion only. However, the subsequent swelling involves the entire cell and results in hemolysis (Fig. 2).

Cells irradiated over their central portions shrink less and hemolyze significantly sooner than those irradiated at their ends. The ends of the Amphiuma erythrocyte are thinner than the center, which contains the nucleus. Since

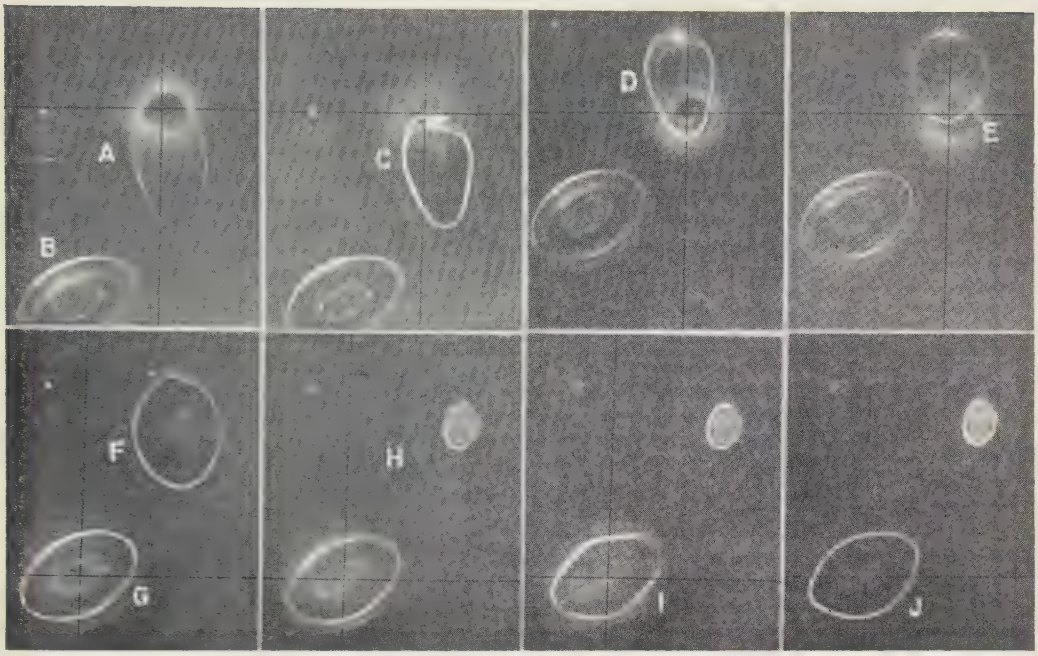


FIG. 2.

Photomicrographs of 2 Amphiuma erythrocytes exposed to partial cell irradiation with polonium alpha particles through a 21.6 $\mu$ hole. Each exposure lasted 90 minutes and corresponded to a dose of 7.5 $\times$ $10^6$ rep. (A) Cell being irradiated at one end; outline of opening through aluminum cap is also shown by focusing down 20 $\mu$ and making a second exposure; (B) normal unirradiated control. (C) 15 minutes later, one end of irradiated cell is severely shrunken; (D) the other end of the same cell is being irradiated. (E) The second end is shrinking; body of cell is swelling. (F) Swelling continues; nuclear change is evident; (G) Control cell is now being irradiated at its center. (H) First cell has hemolyzed 143 minutes after beginning of irradiation. (I) Second cell beginning to shrink, particularly on the lower side 48 minutes after beginning of irradiation; (J) Second cell begins to hemolyze 94 minutes after beginning of irradiation, (complete hemolysis, not shown in figure, occurred 120 minutes after beginning of irradiation).

it is likely that the alpha particles pass completely through the ends of the cells, whereas they do not pass completely through the cell at its center, irradiation at either end of the cell involves twice as much membrane area as is the case for irradiation at the center. It is not clear whether the longer hemolysis time of the cells irradiated at their ends is due to greater shrinkage or to the fact that the nucleus was not irradiated. Experiments are in progress to determine this point.

*Summary.* Erythrocytes from Amphiuma and from man were irradiated *in vitro* with polonium alpha particles (a) from an extended source (4 mm in diameter) and (b) with a small beam (20 $\mu$ in diameter). The cells first shrank, then swelled and hemolyzed. Local irradiation of a part of a single cell resulted in shrinkage only in the irradiated portion. This was followed by swelling of the whole cell and hemolysis.

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# Serologic Differences Among Strains of the Coxsackie Group of Viruses. (17321)

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Since the isolation in suckling mice of an infectious agent from feces of 2 paralyzed children[1–4] a number of fecal specimens from other patients with a tentative diagnosis of poliomyelitis have been examined and 18 additional strains isolated. Some of these differ from the agent originally described in the signs of disease and the lesions they induce in suckling mice, and are being studied. The remaining 11 strains are similar to the original in causing extensive degeneration of the striated muscles of the experimental animal without damage to the central nervous system. In this group 3 serologic types have been encountered.

The localities from which the strains were obtained are indicated in Table I. The first 2 are those originally isolated. Five were obtained from fecal specimens collected in Wilmington, Del., during the 1947 epidemic of poliomyelitis and 6 from cases in 1948 in widely separated communities in New York State.

The serologic differentiation was made by the neutralization test method. Sera were prepared in mice and hamsters. Adult hamsters received living virus, in 10% hamster brain suspension, intraperitoneally in 3 or 4 weekly doses of 1 ml. Mice were given formalinized (strain T.T.) or living virus in mouse brain suspension intraperitoneally in 3 weekly series of 3 daily doses. The amounts injected increased from 0.1 to 0.5 ml. Bleedings were usually taken on the 7th or 8th day after the last dose. All mice and hamster sera were pools. Rabbits were immunized with one strain (J.Ol.). A total of 35.5 to 40.5 ml. of living virus in 10% mouse brain suspension was given intravenously and intraperitoneally over a period of 36 to 46 days. Serum of only one of 3 rabbits was satisfactory. It appeared to be type- rather than species-specific.

Equal amounts of serum and infected suckling mouse brain suspension were mixed and let stand at room temperature for one hour.

TABLE I.
Serologic Types of Strains Isolated in Suckling Mice.

| Patient | Age, yr | Outbreak | | Serologic type |
|---|---|---|---|---|
| | | Year | Locality | |
| T.T. | 9 | 1947 | New York State | 1 |
| K.H. | 3½ | 1947 | " | 1 |
| Fl. Pool | 9,5 | 1947 | Wilmington | 2 |
| M.H. | 13 | 1947 | " | 2 |
| J.K. | 8 | 1947 | " | 2 |
| A.W. | 4 | 1947 | " | 2 |
| C.R. | 12 | 1947 | " | 1 |
| N.C. | 8 | 1948 | New York State | 2 |
| J.H. | 6 | 1948 | " | 2 |
| R.T. | 4 | 1948 | " | 2 |
| L.P. | 4 | 1948 | " | 2 |
| N.A.B. | 4 | 1948 | " | 2 |
| J.Ol. | 2 | 1948 | " | 3 |

1 Dalldorf, Gilbert, and Sickles, Grace M., *Science*, 1948, **108**, 61.

2 Dalldorf, Gilbert, Sickles, Grace M., Plager, Hildegard, and Gifford, Rebecca, *J. Exp. Med.*, 1949, **89**, 567.

3 Gifford, Rebecca, and Dalldorf, Gilbert, Proc. Soc. Exp. Biol. and Med., 1949, **71**, 589.

4 Dalldorf, Gilbert, *Science*, in press.

TABLE II.
Neutralization Tests with Type-3 Strain J.Ol., No. 49,191.

| Undiluted serum | Mouse brain suspension | | | | | |
|---|---|---|---|---|---|---|
| | 10-1* | 10-2 | 10-3 | 10-4 | 10-5 | 10-6 |
| Normal hamster pool, 1/20/49 | | | | 7/7 | 2/7 | 1/7 |
| Type 1, T.T. hamster pool, 11/4/48 | | 8/8 | 8/8 | 8/8 | 5/8 | |
| Normal hamster pool, 6/16/47 | | | | 8/8 | 6/8 | 1/8 |
| Type 2, Fl., hamster pool, 11/8/48 | | 8/8 | 8/8 | 8/8 | | |
| Normal mouse pool, 2/14/49 | | | 8/8 | 8/8 | | |
| Type 3, J.Ol., mouse pool, 3/4/49 | 0/7 | 0/7 | | | | |

* Original dilutions.

Denominator indicates number of mice inoculated; numerator records number dead, paralyzed, or missing during the critical period of the test.

TABLE III.
Effect of Type-3 Rabbit Serum on Strains of 3 Types.

| Undiluted serum | Infected mouse brain suspension | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Type 1, T.T. | | | Type 2, Fl. | | | Type 3, J.Ol. | | | |
| | 10-1* | 10-2 | 10-3 | 10-1 | 10-2 | 10-3 | 10-1 | 10-2 | 10-3 | 10-4 |
| Normal rabbit No. M813, 3/9/49 | 8/8 | 4/7 | 1/6 | 7/7 | 6/6 | 8/8 | | 9/9 | 4/7 | 2/7 |
| J.Ol. immune rabbit No. M813, 5/4/49 | 8/8 | 7/7 | | 7/7 | 7/7 | | 0/6 | 0/7 | | |

* Original dilutions.

Denominator indicates number of mice inoculated; numerator records number dead, paralyzed, or missing during the critical period of the test.

One family of mice was inoculated intraperitoneally (0.05 ml.) with each dose.

One of the 1947 New York State isolations, T.T. (type 1), was selected as the representative strain for serum preparation. Each newly isolated virus was tested for neutralization by this serum. When a strain was encountered that failed to be neutralized, antiserum was prepared with it.

The first Wilmington isolation, Fl., proved to be a second type (type 2) and a 1948 New York State strain, J.Ol., a third type (type 3). Cross-neutralization tests demonstrated a distinct differentiation among the types. Representative tests have been summarized in Tables II and III.

The remaining strains when tested with these sera fell into the types indicated in Table I.

*Summary.* Among 13 virus strains which have been isolated from fecal specimens of patients with a tentative diagnosis of poliomyelitis, and which induce muscle injury in suckling mice, 3 serologic types have been encountered.

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# Gasometric Determination of Hydrogen Peroxide. (17322)

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(Introduced by C. E. Lankford.)

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We[1] have reported the determination of small amounts of peroxide in bacterial culture media by measurement of the oxygen evolution in a Warburg flask when a catalase preparation is added to the peroxide-containing substrate. These determinations were made because the mutagenic properties of irradiated broth have been linked with its peroxide concentration.[2,3] Further work on methods of peroxide determination has shown that the generally accepted overall equation for the breakdown of hydrogen peroxide by the action of catalase,

$$2H_2O_2 \longrightarrow 2H_2O + O_2,$$

does not express the quantitative relationships that occur in the reaction as we employed it. When excess catalase was used to give the rapid completion of the reaction desired for quantitative estimation, the resulting gas output always exceeded the theoretical computed from the above equation.

*Methods.* Known concentrations of hydrogen peroxide were made by dilution of an 82% stock solution, which was checked by iodometric titration, acid permanganate titration, and the colorimetric method of Bonét-Maury.[4] This colorimetric method utilizes a titanium sulfate reagent which gives a yellow color in the presence of $H_2O_2$. A standard curve was prepared by using known concentrations of peroxide in both aqueous and amino acid substrates, and duplicate tubes were found to always agree within $\pm$ 0.1%. The catalase preparations used were catalase "Sarret" (furnished by Vitazyme Laboratories) and a blood preparation extracted according to the method of Sevag.[5] Oxygen evolution was measured in the Warburg respirometer or, when large concentrations of peroxide were used, in a gas burette. The substrate was either water or the amino acid components of the standard synthetic medium for *Micrococcus pyogenes* prepared at 10 times the concentration used for growing the organisms.

*Experimental.* Aliquots of the amino acid solution were irradiated by a quartz ultraviolet light at various temperatures for 20 and 40 minutes and the resulting peroxide concentrations determined by the colorimetric method of Bonét-Maury and by the catalase gasometric method. Table I shows the discrepancies between these two methods. Since the colorimetric method is based on a standard curve made with known concentrations of peroxide and consistently gave accurate results when employed on known samples, it would appear that the gasometric results were in error.

As a further check of the assumed inaccuracy of the gasometric method, known concentrations of $H_2O_2$ were added to water or to the amino acid substrate, and the peroxide concentration indicated by oxygen evolution was calculated. Table II records some typical results.

As a check on experimental methods, known

TABLE I.
$H_2O_2$ Content of Irradiated Amino Acid Solution.

| Irradiation | | ppm $H_2O_2$ | |
|---|---|---|---|
| Temp. | Time, min. | Colorimetric | Gasometric |
| 2.5° | 20 | 4.5 | 6.8 |
| 2.5° | 40 | 7.2 | 16.3 |
| 23° | 20 | 9.0 | 18.1 |
| 23° | 40 | 16.0 | 31.4 |
| 40° | 20 | 9.5 | 19.8 |
| 40° | 40 | 14.2 | 26.2 |
| 58° | 20 | 9.2 | 19.8 |

[1] Wyss, O., Clark, J. B., Haas, F., and Stone, W. S., *J. Bact.*, 1948, **56**, 51.

[2] Stone, W. S., Wyss, O., and Haas, F., *Proc. Nat. Acad. Sci. U.S.*, 1947, **33**, 59.

[3] Wyss, O., Stone, W. S., and Clark, J. B., *J. Bact.*, 1947, **54**, 767.

[4] Bonét-Maury, P., *Compt. rend.*, 1944, **218**, 117.

[5] Sevag, M. G., and Mauvèg, L., *Biochem. Z.*, 1936, **288**, 41.

TABLE II.
Recovery of Hydrogen Peroxide by Gasometric Method.

| Substrate | ppm $H_2O_2$ Added | ppm $H_2O_2$ by $O_2$ evolution |
|---|---|---|
| Water | 8.1 | 17.6 |
| Amino acids | 8.3 | 19.7 |
| Water | 16.8 | 28.6 |
| Amino acids | 16.8 | 28.5 |
| Water | 16,400 | 26,100 |
| Water | 32,800 | 52,400 |

concentrations of $H_2O_2$ were decomposed by acid permanganate and the oxygen evolution measured. This reaction releases one mol of $O_2$ per mol of peroxide and the concentration indicated by $O_2$ evolution always checked very closely with the initial known concentration. Thus the contradictory results are inherent in the catalase-peroxide reaction and not in the experimental method. The gas evolved by the catalase reaction was analyzed and found to be 100% oxygen. As the concentration of enzyme was decreased the total gas evolution decreased to the expected amount, one mol for each 2 mols of $H_2O_2$ decomposed. Further decrease in the enzyme concentration, so that no active catalase could be detected after gas evolution ceased, gave low values for the peroxide content as would be expected from the incomplete reaction.

*Discussion.* The mechanisms postulated for the reaction between hydrogen peroxide and catalase have been discussed by Stern,[6,7] and Sumner,[8] and others. Most of the work done with catalase has been based on the rate of reaction and not the total gas evolved.[9,10] It is obvious from the work by Molland[11] that the reaction is complex and difficult to describe by any simple set of equations. If the mechanism postulated by Keilin and Hartree[10] is correct,

$$4Fe^{+++} + 2H_2O_2 \longrightarrow 4Fe^{++} + 4H^+ + 2O_2$$
$$4Fe^{++} + 4H^+ + O_2 \longrightarrow 4Fe^{+++} + 2H_2O$$
$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

it would appear that under the conditions of our measurements (a well buffered substrate with excess catalase) the second step does not occur. Or if the mechanism postulated by Chance[12] is correct,

$$\text{Cat (OH)}_4 + H_2O_2 \leftrightharpoons [(\text{Cat(OH)}_3\text{(OOH)I} \rightleftharpoons \text{Cat(OH)}_3\text{(OOH)II}]^* + H_2O$$
$$\text{Cat (OH)}_3\text{(OOH)I}^* + H_2O_2 \rightleftharpoons \text{Cat (OH)}_4 + H_2O + O_2$$
$$2H_2O_2 \leftrightharpoons 2H_2O + O_2$$

the reaction does not go to completion under the conditions of our experiments, but stops at one of the intermediate complexes which spontaneously breaks down. It is presumed that the catalase is inactivated in yielding the 1:1 ratio of peroxide to oxygen.

*Summary.* Determinations of the peroxide content of irradiated broth using catalase in a Warburg respirometer were found to be inaccurate. In the presence of excess catalase the oxygen evolved approaches a value of twice that indicated by the accepted general equation for enzymatic decomposition of $H_2O_2$; if too little enzyme is used the reaction is slow and incomplete. The method is unsatisfactory for the quantitative determination of peroxide.

6 Stern, K. G., 1942, A Symposium on Respiratory Enzymes, pp. 74-103, University of Wisconsin Press.

7 Oppenheimer, C., and Stern, K. G., 1939, Biological Oxidation, The Hague.

8 Sumner, J. B., *Adv. Enzymol.*, 1941, **1**, 163.

9 Northrop, J. H., *J. Gen. Physiol.*, 1924, **7**, 373.

10 Keilin, O., and Hartree, E. F., *Proc. Roy. Soc. B.*, 1937, **124**, 397.

11 Molland, J., *Acta Path. et Microbiol. Scand.*, 1947, **66**, 9.

* Intermediate complexes formed.

12 Chance, B., *Nature*, 1948, **161**, 914.

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# Effects of Beta Rays on Central Nervous Tissues.* (17323)

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The present report is made specifically to define the gross morphological changes evoked in the neurons and glial cells of the nervous system by high doses of ionizing radiations. The use of local irradiation by means of a radon applicator was made in order to study a sharp gradient of tissue reaction in the cerebral cortex. Similar technic has been described by Levin[1] in studies of other tissues and by Peyton[2] in an investigation on the effects of radium on the central nervous system. By comparing the various concentric zones of cellular alteration in a lesion the center of which showed complete destruction of cells, the pattern of progressive cytologic alteration has been made clear. In this series of experiments, the high gradient was caused both by the short trajectory of the bombarding electrons in the tissue and by the exponential operation of the law of the inverse square. For the present study, a solution of the difficult question of dosage from radon applicators will be deferred until, in a later report, the effects of dosage will be more fully discussed. Nor will attention be paid in this paper to the possible effects of the very small fraction of ionization caused by the hard gamma radiation which is delivered by the radon applicators. We have no data which would cause one to believe that the action of one type of ionizing radiation is essentially different than of another. The effects of the gamma rays in the present experiments is thought to make the gradient of exposure somewhat less steep by reason of their great tissue penetrating properties. They are, of course, also subject to the law of the inverse square and, forming a small fraction of the total ionization, seem not to complicate matters.

Cats were used in the experiments. Trephining of the calvarium over the parietal region of the cerebral cortex was performed under barbiturate anaesthesia and the dura mater carefully slit and raised. The administration of the irradiation was by means of a small glass bubble (ca 2 mm in diameter) containing emanation of radium. The heads of the cats were held in a rigid holder and the radon applicator was fastened to a 3-directional microdrive and thus directed to the surface of the cortex. The duration of the exposures varied from 5 to 32 minutes, the calculated activity of the applicators from 30 to 78 millicuries. The dura was then returned to place (in the longer experiments) and the skin carefully sutured over. Killing was by overdose of sodium pentobarbital. Where imprints of the lesions were made, the cortex was dissected out fresh and after the imprints, the material of the lesion was fixed by immersion in 10% formalin. In several of the experiments, the animal was first perfused with formalin and the lesions subsequently dissected out. Though maps of the sulci and the positions of the sites of irradiation were kept, some difficulty was had in locating those lesions which were not strongly enough irradiated to cause a visible pink spot.

*Observations.* Fig. 1 shows the edge of a typical lesion in the cerebral cortex 5 hours after exposure. To the right is the area, just visible in the photograph, of complete destruction. Towards the left the affected area borders the normal cortex. The alteration towards basophily of the neurons and of the glia is visible. The meninges and the surface of the brain frequently are much altered at the point of contact with the applicator. In all of the marked lesions, as in Fig. 1, there was considerable breakdown of the layered structure of the cerebral cortex. The disturbance of the tissue seldom extended into the neighboring white matter. The neuronal changes in the area bordering that of complete tissue

* Aided by a grant from The National Foundation for Infantile Paralysis.

1 Levin, Proc. Soc. Exp. Biol. and Med., 1924, **21**, 462.

2 Peyton, W., *Am. J. Cancer*, 1934, **20**, 558.

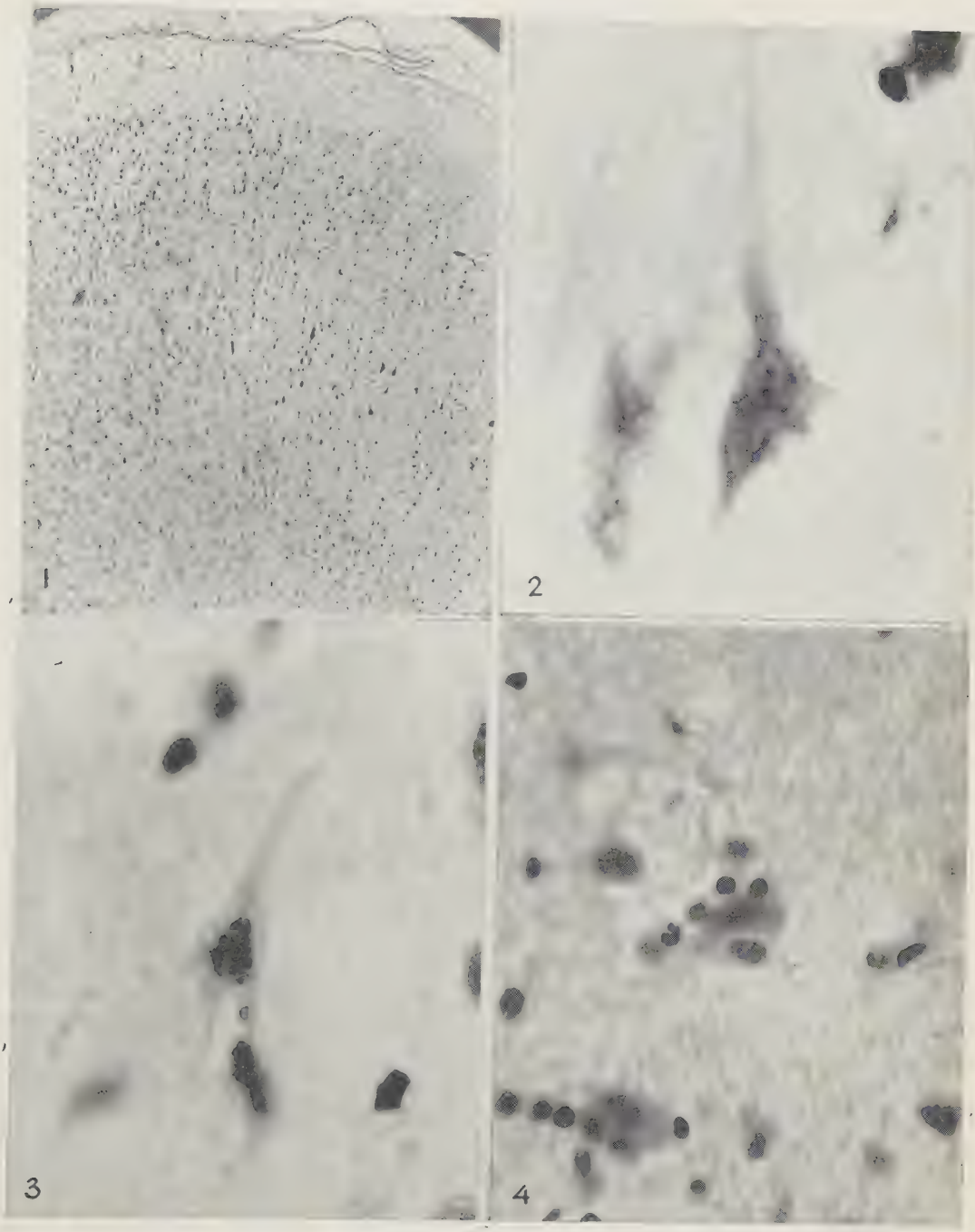


FIG. 1. Edge of small lesion in cerebral cortex 5 hours after irradiation. To the right is the area of heavy destruction, to the left that of unaffected tissue. Cresyl violet stain.

FIG. 2. Hyperchromic nerve cell of fifth cell layer of cerebral cortex. Cresyl violet stain.

FIG. 3. "Polka-dot" degeneration of cell from fifth cell layer of cerebral cortex. Cresyl violet stain.

FIG. 4. Plasma cell like changes in satellite oligodendroglia in fifth cell layer of cerebral cortex. Hematoxylin and eosin stain.

destruction are severe and segregate into two general patterns. First is the hyperchromic reaction which leads to cells similar to those described by Miller[3] and Hartmann[4] in cerebral cortex of cats and other animals. This is characterized by a simultaneous increase in basophilia of all elements of the cell and a shrunken, ragged appearance. The second type of change observed leads to fragmentation of the nuclear chromatin and a progressive loss of nuclear membrane and cytoplasmic structure. In the area of heaviest irradiation all of the neurons of the cerebral cortex are affected. Toward the edge of the lesions it is seen the changes continue for a greater distance from the source in the large pyramidal cells of Layer 5 than in the granule cells of Layer 4. Inspection of the deeper layers closer to the center of the lesion shows that the large pyramidal cells, which seem affected by a lower dosage, also show a greater resistance to the rays in that they persist, as hyperchromic cells, in areas quite devoid of other neuron types. Further towards the center of the lesion, they, too, suffer dissolution.

The hyperchromic cells are conspicuous in Fig. 1. In Fig. 2 is shown a large pyramidal cell from Layer 5 with a long apical dendrite. All of the dendrites are deeply stained, but the cork-screw appearance mentioned by Miller and Hartmann[3,4] is not conspicuous. The margin of the cytoplasm is heavily vacuolated, giving the cell body a scalloped effect. The Nissl bodies are visible and discrete and of normal appearance except for the intense affinity for basic stains. The cytoplasmic matrix (the unstainable substance of Nissl), which is usually refractory to staining by most dyes, is heavily colored by the basic stain and in many of the more pycnotic cells completely occludes the finer structure of the cell. In the example seen in Fig. 2, the nucleus contains a characteristically hypertrophied nucleolus. In many instances, the nucleus has the appearance of being filled with nucleolar material. The nuclear membrane is usually heavy and covered with small densely staining plaques of chromatin. The axons are not stained.

More common than the hyperchromic alterations in the areas of heavy irradiation are those cells showing fragmentation of the nucleus and dissolution of the cytoplasm as their main features. In these cells, both the Nissl bodies and the rest of the cytoplasm have very little affinity for the stain and the nuclear changes may be followed with considerable ease. There is an increase in the amount of chromatin in the nucleus and it is seen to assume a pattern quite unlike that seen in neurons under other conditions. Heavy rounded accumulations of chromatin are formed giving the nucleus a "polka dot" appearance. In the cells least altered, as in Fig. 3, this nuclear material is of considerable bulk and a thin nuclear membrane may be made out. Other cells, apparently more affected, show loss of size of the granules and a thinning of the nuclear membrane. The nucleolus disappears. The cytoplasm of the more affected cells as well as the cell membranes are visualized only with difficulty or not at all, and near the center of the lesion remains of the neurons consist merely of an accumulation of small heavily staining granules.

The small lesions produced by these experiments are especially useful in studying the glial responses to irradiation. Across the steep gradient from normal tissue to the necrotic center of the lesion (a distance of only a few millimeters) the changes brought about by a full spectrum of adequate dosage may be observed. The fate of the astrocytes, as traced in these lesions is that of slight nuclear swelling followed by dissolution. No cytoplasm is visible in any of the stages. The nuclear material becomes finely stippled in the terminal stages, reminiscent of the dotted chromatin pattern of some of the neurons. The position in the lesion where the astrocyte may last be recognized indicates that it is more radiosensitive than either the neurons or the other glial cells.

The oligodendroglia and the microglia, whose naked nuclei may be quite well distinguished in the normal appearing periphery of the lesion show convergent changes as

[3] Miller, R., *Am. J. Anat.*, 1949, **84**, 201.

[4] Hartmann, F. P., unpublished manuscript.

they are followed through the zones of higher exposure. These consist of a marked hypertrophy of the nuclear membranes and the accumulation of visible cytoplasm. There is no sign of either cell undergoing destruction in the outer zones and the phenomenon of convergence to a common cell type is well marked. In the region of intense destruction of the neurons, these altered cells of the mesoglia show a tendency to clump around the remains of the nerve cells but signs of phagocytic activity were not made out with certainty (Fig. 4). Instead, the cytoplasm showed an absence of the foamy and detritus ladened appearance of true macrophages. Examination of these altered glial cells in air-dried imprints of the lesions stained with Wright-Giemsa shows that the cells resemble, in some particulars, the brain plasma cell. The nuclei tend to be eccentric, the chromatin particles very coarse. There is a basophilic cytoplasm which resembles in its staining and texture that of the plasma cell. A perinuclear halo is, however, infrequently seen.

The changes undergone by these mesoglial cells (cligodendroglia and microglia) are similar to those of other cells of supportive tissue in inflammatory reactions. Thus the nucleolar chromatin increases in amount, the nuclear membrane hypertrophies, and both the volume and the nucleic acid content of the cytoplasm is altered upward. As is the rule in other supporting tissues, the rounding up of the cells occurs as a correlate of these processes.

In the older lesions, infiltration of hematogenous cells, first polymorphonuclear leucocytes and later lymphocytes occurs. The former species degenerate, the lymphocytes differentiate into intermediate polyblasts as described by Good.[5]

*Discussion.* A limiting factor in the interpretation of results such as presented here is the impossibility of ruling out the indirect effect of the irradiation on the cell components of nervous tissue by disturbance of the vascular system. Work is underway to circumvent the complication by irradiation of cells in tissue culture. Prior to such improved data, however, the present findings are not without considerable interest.

The ability of such small radioactive sources to make restricted lesions in the nervous system has obvious uses. Not only does the small size of the lesion furnish a gradient from normal to destroyed tissue along which changes may be traced with especially good control, but bloodless destruction of certain tracts and nuclei whose vascular system is related to surface vessels may be more practical than hitherto. Details necessary for exploitation of this method in surgery must be worked out in long term experiments of this sort.

The chromophilic "sclerotic" nerve cell, so conspicuous in these preparations, has been previously studied in this laboratory (Miller,[3] Hartmann[4]) and found to be more than a fixation artifact. Production of this cell change by such means as ischemia (Tureen[6]), inanition, or chronic methylene blue poisoning (Näätänen[7]) has not been successfully duplicated in this laboratory.

Both the changes in the neurons and in the glia show the protein synthetic mechanisms of the cells to be susceptible to alteration by ionizing irradiation. The as yet undeciphered link between the genic chromatin in the nucleus, the cytoplasmic (and nucleolar) ribose nucleic acid, and physiological states associated with inflammation is apparently a target in the experiments described here. Not only in the chromophilic neuron, but particularly in the glia, is this obvious. The mesoglial elements give rise to plasma-cell like cells rather than to macrophages as would be expected in lesions of similar magnitude formed through other means. While these cells show some tendency to cluster around the neurons, phagocytic activity, as judged by the appearance of the cytoplasm, could not be postulated. As recent work on the relations of macrophages and plasma cells in experimental brain lesions has shown that

[5] Good, R. A., *J. Neuropath. and Exp. Neurol.*, in press.

[6] Tureen, L. L., *Arch. Neurol. and Phychiat.*, 1936, **35**, 789.

[7] Näätänen, E., *Acta path. et microbiol. Scand.*, 1945, **22**, 603.

the functions of secretion of antibodies and of scavenging are sharply differentiated,[5] the most reasonable interpretation is that these destructive ionizing radiations have produced aberrant secretory cells, possibly analogous to the neoplastic cell in multiple myeloma.

The phenomenon of convergence of the oligodendroglia and of the microglia seen in these preparations is further evidence of the thesis supported by the senior author[8,9] that the microglia and the oligondendroglia are functional states of the same cell type and that both contribute to the formation of inflammatory cells in encephalitic disease. The clarity with which this process can be seen in the transition zones of the beta ray lesions makes the demonstration of similar evolution of the two species of mesoglia more obvious than any other preparation. That it is not simply a particular response to this highly unusual stimulation is substantiated by the parallelism of the changes in herpetic virus disease.

*Summary.* 1. The application of small, high-energy, applicators of radium emanation to the cerebral cortex of cats produces restricted lesions in which progressive changes of the cells of that tissue may be followed from normal appearing tissue to that showing complete necrosis over a distance of several millimeters.

2. Nerve cells so affected show two types of destruction. One involves lysis of the cytoplasmic structures and degeneration of the nucleus into a small aggregation of basophilic granules. The other consists of chromophilic alteration of the cells. The pyramidal cells of layers 2, 3, and 5 seem especially susceptible to this change. The cells become shrunken, extremely heavy staining, and show hypertrophy of the nucleolus.

3. The astrocytes are the most susceptible of the various cells of the cerebral cortex. They undergo lysis in the middle zones of the lesions.

4. The mesoglial cells, oligodendroglia and microglia, are highly resistant. They show progressive changes, from the edge to the center of the lesions, which by convergence, make the two types indistinguishable. The product of these changes is a plasma-cell like structure which shows no phagocytic activity.

8 Campbell, B., *Anat. Rec.*, 1947, **97** (Suppl.), 7.

9 Campbell, B., *J. Neuropath. and Exp. Neurol.*, 1949, **8**, 347.

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## Lysozyme Content of Granulation Tissue.* (17324)

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The highest lysozyme concentrations in the mammalian body occur in tears and in the mucosa of the antrum, pylorus, and duodenum.[1] The origin of these high local concentrations presumably is epithelium, *i.e.*, the tear glands and as yet undetermined cell types in the gastrointestinal mucosa. In contrast, the lysozyme titer in mesodermal tissue is low; for example, serum averages 1 unit/cc,[1] and human leucocytes (from the buffy coat of normal blood) contain only 1.8 units per 5,000,000 cells.[2] However, human cartilage averages about 40 units/g, although this value is without doubt too low because of the difficulty in extracting this tissue. Normal human skin (including a considerable quantity of fibrous tissue) was found to have less than 1 unit/g.

The finding of high lysozyme titres in gran-

* Supported in part by the Research Grants Division of the U. S. Public Health Service, and the Josiah Macy, Jr. Foundation.

1 Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am. J. Med.*, 1948, **5**, 482.

2 Meyer, K., in Modern Trends in Ophthalmology, 2:71, Paul B. Hoeber, New York, 1947.

TABLE I.

| Granulation tissue | Titre in units/g |
|---|---|
| A. Tissue from experimental granulating canine wounds | |
| 1. Dog. No. 1 Age of wound | |
| 3 days | 80 |
| 8 " | 108 |
| 14 " | 208 |
| 2. Dog No. 2 | |
| a. from center of wound, 14 days | 77 |
| b. from periphery—mostly new epithelium, 14 days | 10 |
| B. Tissue from granulating human wounds | |
| 1. Indolent ulcer from pyoderma gangrenosum in chronic ulcerative colitis; 2 occasions | 23; 30 |
| †2. Same wound after healing had begun (chemotherapy) | 170 |
| †3. Granulating dog bite in a human (1 week) | 224 |
| 4. Indolent varicose ulcer | 91 |
| 5. " " " | 81 |
| †6. Granulating thoracotomy wound | 564 |
| †7. Granulating laparotomy wound | 161 |
| 8. Necrotic granulations from laparotomy wound | 500 |
| †9. Infected laparotomy wound | 91 |
| †10. " " " | 118 |
| †11. " " " | 159 |
| C. Rabbit callus determinations in fractured femur | |
| 1. 5 days | 84 |
| 2. 8 " | 60 |
| 3. 14 " | 16 |

† Healthy appearing granulating tissue.

ulation tissue was therefore unexpected. The lysozyme assays on granulation tissue of man and dog are shown in the accompanying table.

The assays were done by a viscosimetric method[4] on extracts prepared as previously described.[3]

As a result of these observations it is now apparent that high lysozyme concentrations are associated with some mesodermal cell types as well as with epithelium. Therefore, further study is warranted with regard to the role of this tissue in the production of lysozyme in ulcerative alimentary disease.

The deleterious effect of egg white lysozyme on the gastrointestinal mucosa[1,3] has been confirmed in our own[5] as well as other laboratories.[6,7] Furthermore, high stool titres in the absence of occult blood in the feces and with sigmoidoscopically non-ulcerated mucosa are frequently observed in chronic ulcerative colitis. These two considerations render less likely the possibility that granulation tissue is the source of the major fraction of the lysozyme titre in ulcerative alimentary disease.

[3] Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 220.

[4] Meyer, K., and Hahnel, E., *J. Biol. Chem.*, 1946, **163**, 723.

[5] Prudden, J. F., Lane, N., and Meyer, K., to be published.

[6] Grossman, M. I., personal communication.

[7] Grace, W. J., Seton, D. H., Wolf, S., and Wolff, H. G., *Am. J. Med. Sci.*, 1949, **217**, 241.

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## Occurrence of Conjugase in Egg Yolks.* (17325)

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Pteroylglutamic acid conjugases are enzymes that liberate microbiologically available PGA from more complex forms of the vitamin and have been reported in hog kidney,[1] chicken pancreas,[2,3] rat liver[4] and blood.[5] During the course of routine PGA determina-

* This work was supported in part by a grant from the Lederle Laboratories Division, American Cyanamid Company.

[1] Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. Biol. Chem.*, 1945, **159**, 631.

TABLE I.
Pteroylglutamic Acid Values Obtained by Various Treatments of Egg Yolk.*

| Treatment of sample | Autoclaved† | Not autoclaved | Autoclaved + chick pancreas | Not autoclaved + chick pancreas | Not autoclaved + autoclaved chick pancreas |
|---|---|---|---|---|---|
| | Micrograms of PGA per 100 g of egg yolk (fresh weight basis) | | | | |
| Chicken egg yolk | 10.2 | 31.9 | 35.3 | 81.4 | 16.3 |
| Turkey egg yolk | 41.9 | 60.2 | 62.9 | 129.7 | 60.8 |

* Each value in this table is the average of seven separate assays.
† All samples were incubated at 37.5° C for 24 hours.

tions in the laboratory, an indication was obtained that egg yolk also contains such a conjugase and the present study was initiated to determine if this was correct.

*Experimental.* In order to test this hypothesis, the following treatments of samples were used. In the first place the sample was autoclaved for 15 minutes at 15 lb pressure, incubated for 24 hours at 37.5°C and assayed for free PGA. In the second treatment the sample was not autoclaved but was incubated in an effort to determine if any conjugase was present in the egg yolk. In the third treatment the sample was autoclaved and 20 mg of dried chick pancreas was added prior to incubation of the sample, in order to ascertain the amount of PGA liberated by the conjugase of chick pancreas. The fourth treatment consisted of adding chick pancreas to the unautoclaved egg yolk sample in order to determine total PGA and finally 20 mg of autoclaved chick pancreas was added to a sample of egg which had not been autoclaved in order to determine if egg yolk conjugase would liberate PGA from chick pancreas. The tentative assay procedure for PGA, as devised by Flynn[6] for AOAC collaborators, was used in determining the microbiologically available PGA with *Lactobacillus casei* as the test organism; growth was measured by acidimetry. According to this procedure the sample is autoclaved first and then chicken pancreas is added prior to incubation.

Chicken egg yolks used in these studies were taken from eggs laid by Single Comb White Leghorn pullets that had been in production for approximately 4 months. The turkey egg yolks were obtained from eggs laid by Broadbreasted Bronze turkey pullets that were just beginning to lay. Both the turkeys and chickens were fed practical all-mash diets and were maintained in individual laying cages with raised screen bottoms. All eggs were collected daily and placed in a refrigerator at a temperature of approximately 4.4°C. In each case the pteroylglutamic acid determination was carried out on an individual egg yolk. The egg was broken and the white was separated and discarded. A 10 g sample of the fresh yolk was blended in the Waring blendor with 90 ml of distilled water. A 10 ml aliquot was pipetted into a 125 ml Erlenmeyer flask. Thirty ml of phosphate buffer (pH 7.0) and 10 ml of a chick pancreas solution, which contained 2 mg dried chick pancreas per ml, were added. The dried chick pancreas was prepared according to the method of Burkholder, McVeigh and Wilson.[7] The treatment of the samples prior to incubation is indicated in Table I. All samples were covered with a layer of toluene and incubated at 37.5°C for 24 hours. After incubation the samples were autoclaved for 15 minutes at 15 lb pressure, diluted to 100 ml, filtered and aliquots were taken from this stock solution for dilution purposes.

When the egg yolk sample was autoclaved for 15 minutes at 15 lb pressure and incubated it was assumed that any enzymes which

[2] Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1945, **157**, 731.

[3] Mims, V., and Laskowski, M., *J. Biol. Chem.*, 1945, **160**, 493.

[4] Sreenivasan, A., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 117.

[5] Simpson, R. E., and Schweigert, B. S., *Arch. Biochem.*, 1949, **20**, 32.

[6] Flynn, L., Mimeographed Report to Collaborators on Folic Acid, 1948.

[7] Burkholder, P. R., McVeigh, I., and Wilson, K., *Arch. Biochem.*, 1945, **7**, 287.

TABLE II.
Liberation of PGA from Egg Yolk by Different Conjugases.

| Liberation by different conjugases | Free PGA | PGA liberated by egg conjugase | PGA liberated by chick pancreas conjugase | Total PGA | PGA liberated by synergistic action of egg yolk and chick pancreas conjugase | PGA liberated by egg conjugase from chick pancreas |
|---|---|---|---|---|---|---|
| | Micrograms of PGA per 100 g of egg yolk (fresh weight basis) | | | | | |
| Chicken egg yolk | 10.2 | 21.7 | 25.1 | 81.4 | 24.4 | —15.6 |
| Turkey egg yolk | 41.9 | 18.3 | 21.0 | 129.7 | 48.5 | +0.6 |

were present were destroyed. Free PGA determined on such a sample amounted to 10.2 μg per 100 g of chicken egg yolk (Tables I and II). The free PGA value for turkey egg yolk was about 4 times that of the chicken egg yolk.

The incubation of samples of egg yolk that had not been autoclaved resulted in an increase in PGA over that of the autoclaved sample described above (Tables I and II). The magnitude of this increase was about the same for both the chicken and turkey egg yolks and amounted to 21.7 μg PGA per 100 g yolk in the chicken egg and to 18.3 μg per 100 g in the turkey egg yolk (Table II). From these results it is apparent that egg yolk from the chickens and turkey contains a conjugase which liberates microbiologically available PGA.

When the sample was autoclaved and chick pancreas added as outlined in Flynn's procedure[6] this source of conjugase liberated 21.0 μg of PGA per 100 g of chicken egg yolk and 25.1 μg of PGA per 100 g of turkey egg yolk (Table II). These values are increases above the free PGA values and must have resulted through the action of chicken pancreas conjugase. The value obtained for chicken egg yolk where the sample was autoclaved and chick pancreas added (Table I) is in the range of that obtained by the AOAC collaborators (Flynn[6]) when it is converted to a dry weight basis.

The total PGA, determined by adding 20 mg of chick pancreas to the unautoclaved sample, was 81.0 μg per 100 g for the chicken egg yolk and 129.7 μg PGA per 100 g of turkey egg yolk. These figures are higher than that which can be calculated by adding free PGA, PGA liberated by egg yolk conjugase and that liberated by chick pancreas conjugase (Table II). A total of these values gives 57.0 (10.2 + 21.7 + 25.1) μg PGA per 100 g chicken egg yolk and 81.2 (41.9 + 18.3 + 21.0) μg per 100 g turkey egg yolk. Thus 24.4 and 48.5 μg PGA per 100 g egg yolk (Table II) were liberated by the synergistic action of egg yolk and chicken pancreas conjugases for the chicken and turkey egg yolks respectively. The total PGA value obtained for chicken egg yolk when converted to the dry weight basis approaches the lower value for PGA obtained by Flynn and collaborators[6] when dried egg yolk was assayed for PGA by the chick assay. Thus with the combined action of egg yolk conjugase and chick pancreas conjugase the value for the microbiologically available PGA approaches a similar value obtained by means of the chick assay for this vitamin.

One sample was tested in each series to determine whether egg yolk conjugase was liberating PGA from the chick pancreas. In this instance the chick pancreas solution (2 mg per ml) was autoclaved for 15 minutes at 15 lb pressure and added to the unautoclaved egg yolk. The results (Table I) show that no appreciable liberation of PGA from chick pancreas was obtained by turkey egg yolk conjugase. A slight inhibitory effect was noted on the conjugase in chicken egg yolk when autoclaved chick pancreas was added prior to incubation. The PGA liberated by egg yolk conjugase amounted to only 6.1 μg per 100 g; whereas, 21.7 μg PGA was liberated by egg yolk conjugase when autoclaved chick pancreas was not added. This represents a decrease of 15.6 μg PGA per 100 g yolk in

the presence of autoclaved chick pancreas (Table II).

From the results of this study maximum liberation of PGA from egg yolk occurs when egg yolk conjugase and chick pancreas conjugase act together.

*Summary.* Evidence is presented to show that chicken and turkey egg yolk contain a conjugase which liberates PGA from these materials. A synergistic action of egg yolk conjugase and chick pancreas is required in order to obtain the total PGA in egg yolk of chickens and turkeys. The liberation of microbiologically available PGA from egg yolk by the combined action of egg yolk conjugase and chicken pancreas conjugase gives a total PGA value which approaches the value obtained by the chick assay for this vitamin. An inhibitory effect of autoclaved chick pancreas on the conjugase of chicken egg yolk is indicated by the results.

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## Effect of Thyroxine on Lymphoid Tissue Mass of Adult Male Mice.* (17326)

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That the secretion of the thyroid gland is intimately related to the physiology of lymphoid tissue has been suggested by both clinical observation and laboratory experiment. Marine and co-workers[1] emphasized that peripheral lymphadenopathy, splenomegaly, and persistence of the thymus gland were detectable in many patients with Graves' disease. Several investigators[2-4] have reported that stimuli which cause accidental involution of the lymphoid tissue of laboratory animals may have a more pronounced or more prolonged effect in the absence of the thyroid gland.

An important link in the chain of evidence —namely that augmented thyroid secretion results in hyperplasia of lymphoid tissue— has previously evaded demonstration despite several experimental trials.[3,5] One possible explanation for this failure lies in the fact that the rat was the experimental animal employed in most of these investigations. Evidence is accumulating that this animal, in the resting state, may be secreting thyroid hormone at a high rate;[6] thus the addition of small doses of exogenous hormone might well be without significant effect. Large doses of hormone may produce relative malnutrition and involution, rather than hyperplasia, of lymphoid tissue.[7] It was, therefore, considered desirable to observe the consequences of thyroxine treatment in the mouse. Profound changes in the lymphoid tissues were observed. In view of the known alterations in adrenal cortical activity following treatment with thyroid hormone,[8,9] a parallel experiment was conducted in adrenalectomized animals to study the possible contribution of this organ

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† Post-doctorate Research Fellow, National Cancer Institute, United States Public Health Service.

1 Marine, D., Manley, O. T., and Bauman, E. J., *J. Exp. Med.*, 1924, **40**, 429.

2 Selye, H., *Endocrinol.*, 1937, **21**, 169.

3 Reinhardt, W. O., and Wainman, P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 257.

4 White, A., and Dougherty, T. F., *Endocrinol.*, 1947, **41**, 230.

5 Korenchevsky, V., and Hall, K., *Biochem. J.*, 1941, **35**, 726.

6 Meites, J., and Chandrashaker, B., *Endocrinol.*, 1949, **44**, 368.

7 Andreasen, E., *Acta path. et microbiol. Scand.*, 1939, **15**, 259.

8 Lowenstein, B. E., and Zwemer, R. L., *Endocrinol.*, 1943, **33**, 36.

9 Deane, H. W., and Greep, R. O., *Endocrinol.*, 1947, **41**, 243.

to the changes observed in the intact animal.

*Methods.* Adult male mice of strain CBA (Strong) were employed. Littermate pairs were assigned to one of two parallel experiments. In the first, which tested the action of thyroxine in intact animals, one member of each pair was assigned at random to the treatment group while its littermate served as a control. In the second experiment, all animals were adrenalectomized, and again the pairs were divided at random into thyroxine-treated and control groups.

Until they reached the age of 11 weeks, the animals were raised on Purina Laboratory Chow and tap water. The diet was then changed to one of bread and milk to which was added whole dried Brewers' Yeast to 5% by dry weight. Both diets were fed *ad libitum*.

Treatment was begun when the animals were 13 weeks of age. The experimental animals received daily intraperitoneal injections of 15 $\gamma$ of thyroxine‡ in 0.1 ml of slightly alkaline distilled water. Controls received an equal volume of solvent prepared exactly as that employed for the thyroxine. The treatment period was 21 days for the intact animals; 14 days for the operated animals.

Bilateral adrenalectomy was performed in one stage via the lumbar route. Ether and light nembutal anesthesia were employed. For the first 3 post-operative days, 5% glucose and 0.9% sodium chloride were added to the drinking water of the operated animals. For the remainder of the experimental period, the drinking water contained only 0.9% sodium chloride. Operations were performed when the animals were 12 weeks of age; adrenalectomized animals were thus on the experimental diet for one week preoperatively, and were allowed a post-operative interval of one week before thyroxine therapy was instituted.

Eighteen hours after the last injection, the animals were killed by cervical dislocation and complete autopsies carried out. These were performed in a routine manner and included, in addition to the general examination, an inspection of the operative site for post-operative infection or evidence of regenerated adrenal remnants or hypertrophied accessory adrenal tissue. The following organs were dissected free of surrounding tissue and weighed on a precision torsion balance: both adrenals, both kidneys, the spleen, the thymus gland, and a sample of the peripheral lymph nodes which consisted of the two axillary nodes from each side and the two epigastric nodes. Body weights were measured on a double-beam balance at the beginning of therapy and at autopsy.

Statistical analysis involved the application of Fisher's "t-test" for small groups. In this report the word "significant" will be used only to describe differences wherein the analysis has indicated a probability value of the order of 0.01 or less.

*Results.* The results of the two experiments are collected in Table I. Since body weights do not differ significantly, absolute rather than relative values are given. For the adrenal glands, the range of the weights rather than the standard error is noted as the experimental error is thought to be greater than that due to sampling.

The administration of thyroxine to intact animals resulted in a significant increase in the weights of the spleen, peripheral lymph nodes, and kidneys. A suggestive rise in adrenal weights was observed. The weights of the thymus glands of the treated animals were less than those of their controls. While this difference amounts to almost 20% of the control values, and has a p-value of less than 0.01, the results must be regarded with caution as the sampling error is slightly less than the error generally accepted for the weights of dissected organs ($\pm$ 1 mg).

The reduced tolerance of adrenalectomized animals to thyroid hormone[10] was illustrated by the fact that 7 of 17 animals so treated died within 2 weeks, while all but one of the control group survived. Because of this serious loss, the experiment was terminated after a treatment period of 14 days. The results reported are those obtained for the littermate pairs in which both members survived this

‡ l-thyroxine, Squibb.

[10] Koelsche, G. A., and Kendall, E. C., *Am. J. Physiol.*, 1935, **113**, 335.

TABLE I.
Body Weights and Organ Weights of Adult Male Mice Treated with Thyroxine.

| Group | No. of mice | Body wt (g) | | Organ wt (mg) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Initial | Final | Spleen | Lymph nodes | Thymus | Kidneys | Adrenals |
| Intact Control | 14 | 28.3 ±0.5 | 29.7 ±0.5 | 103 ±3.4 | 32 ±1.2 | 27 ±0.9 | 470 ±9.6 | 4 (3-5)‡ |
| Intact Thyroxine* | 14 | 28.1 ±0.5 | 29.5 ±0.5 | 170 ±8.2 | 53 ±4.1 | 22 ±0.9 | 586 ±13.3 | 5 (4-6)‡ |
| Adrenalectomized Control | 9 | 27.4 ±0.6 | 28.3 ±0.6 | 161 ±5.3 | 47 ±1.8 | 45 ±1.2 | 441 ±10.8 | — — |
| Adrenalectomized Thyroxine† | 9 | 27.3 ±0.6 | 28.2 ±0.7 | 224 ±5.6 | 61 ±3.2 | 53 ±3.5 | 500 ±27.6 | — — |

* 15 γ per day for 21 days.
† 15 γ per day for 14 days.

‡ Range of values. Other values are means and standard error by $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

length of time. These results illustrate the well-known effects of adrenalectomy on lymphoid tissues. In addition the weights of the spleen and lymph nodes of the thyroxine-treated animals are significantly higher than those of their controls. The thymus weights showed an increase of limited significance ($p = <0.05$). The shorter course of thyroxine treatment employed for this group did not result in a significant change in kidney weights ($p = >0.05$). Body weight values demonstrate that the dietary regimen adequately maintained the operated animals; and the stability of these values indicates that the animals are well past the period of most active growth (4 to 8 weeks).[11]

*Discussion.* While quantitative histologic studies were not performed, microscopic examination suggested that much of the increase in lymphoid tissue mass was due to hyperplasia of these organs. In response to either thyroxine or adrenalectomy, the spleen and peripheral nodes registered an increase in weight of over 50%. The two treatments appeared to be additive and together resulted in a doubling of the weights of these organs. That this increase following thyroxine treatment occurred in animals showing essentially stable body weights indicates that the effect of this treatment was quite distinct from any that might result from a change in rate of growth.[12] Furthermore, exhaustion of the adrenal cortex[9] does not appear to have contributed to this effect, since treatment of adrenalectomized animals with thyroxine resulted in an increased weight of the spleen and peripheral nodes over and above that following adrenalectomy alone. If anything, the data for the adrenal and thymic weights of the first experiment suggest augmented adrenal cortical activity during the period studied. The slight decrease in thymic weights of intact animals treated with thyroxine appears not to have occurred in the adrenalectomized animals. This behavior of the thymus, in contrast to the other lymphoid tissues studied, is consistent with its greater sensitivity to the action of adrenotrophic hormone.[13,14]

An increase in kidney weight has been previously shown to follow thyroid treatment in rats[15] and testosterone treatment in mice.[16] In both instances, the renal hypertrophy was

[11] Morris, H. P., *J. Nat. Cancer Inst.*, 1944, **5**, 115.

[12] Koger, M., Hurst, V., and Turner, C. W., *Endocrinol.*, 1942, **32**, 237.

[13] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

[14] Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 135.

[15] MacKay, E. M., and MacKay, L. L., *J. Nutrition*, 1931, **4**, 33.

related to an increased rate of protein metabolism. It is perhaps significant that adrenal cortical hypertrophy, well known to occur under thyroid treatment, is also thought to be a response to an increased rate of protein utilization.[17] In addition, evidence has recently been presented that lymphoid tissue may represent an important source of metabolic protein[4]—a source limited in volume, but of extreme lability. While the present experiments record only gross structural alterations, and the possible contribution of these changes to the physiology of the organism must remain in the realm of speculation, it is suggested that an increased rate of nitrogen metabolism may well represent the common denominator underlying the many changes noted in this investigation.

*Summary.* The administration of thyroxine to adult male mice resulted in an increase in the organ weights of the kidneys, spleen, and peripheral lymph nodes. Similar treatment of adrenalectomized animals resulted in an increase in the weights of the spleen and peripheral lymph nodes over and above that which followed adrenalectomy alone. It is concluded that thyroxine produces an increase of lymphoid tissue mass which is independent of the rate of body growth and of the level of adrenal cortical activity.

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[16] Kochakian, D. C., in Recent Progress in Hormone Research, Vol. 1, New York, Academic Press, Inc., 1947, p. 177.

[17] Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinol.*, 1943, **32**, 373.

## Failure of Aureomycin in the Treatment of Experimental Tuberculosis. (17327)

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The failure of streptomycin, p-aminosalicylic acid and the sulfone compounds to prove ideal agents for the treatment of tuberculosis has stimulated the testing of many new agents. Because of the remarkably wide anti-microbial spectrum of aureomycin, it seemed worthwhile to study its effect upon experimental tuberculosis.

Guinea pigs weighing 400 to 500 G each were inoculated subcutaneously with 0.02 mg tubercle bacilli from a recently isolated, virulent, streptomycin-sensitive human strain. After 28 days, the animals were divided into two groups. The 12 animals in Group A were treated with daily subcutaneous injection of 5 mg aureomycin hydrochloride, 6 days a week for 6 weeks. The 11 animals in Group B served as infected, untreated controls.

A third group, Group C, consisted of 6 uninfected guinea pigs given aureomycin in the same dosage as Group A, in order to observe possible toxic effects of the drug.

Animals were autopsied as they died, and those surviving after 6 weeks of treatment or 10 weeks after infection were sacrificed and autopsied.

In Table I, it can be seen that mortality was higher in the treated than in the untreated group. Both treated infected and uninfected groups showed marked weight loss, and one of the uninfected animals died during treatment.

All the infected animals showed widespread tuberculous involvement of the regional lymph nodes surrounding the injection site, the liver, spleen, and lungs. Gross and microscopic examination failed to show any appreciable difference between the treated and untreated animals. Acid fast bacilli were demonstrated

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TABLE I.
Effect of Aureomycin on Weight and Survival of Tuberculous Guinea Pigs.

| Group | Avg wt start, g | Avg wt start treatment, g | Avg wt death, g | No. animals start | No. animals surviving 10 wks |
|---|---|---|---|---|---|
| A. Infected, aureomycin treated | 501 | 556 | 440 | 12 | 5 |
| B. Infected, untreated | 471 | 529 | 552 | 11 | 10 |
| C. Uninfected aureomycin treated | 465 | 652 | 578 | 6 | 5 |

in the microscopic sections of tuberculous tissue from the 5 animals which survived the full 6 weeks of treatment, and tubercle bacilli were readily cultured from the lung in one of these animals.

All the animals treated with aureomycin showed definite evidence of local drug toxicity. There was marked fibrosis of the anterior abdominal wall, where the injections were given, and there were extensive peritoneal adhesions, which, in a few animals, constricted and partially obstructed loops of small intestine. This mechanical obstruction may have accounted for the weight loss in some of the treated animals. In 6 of the treated animals, intercellular material that stained blue with hematoxylin was observed in the liver, spleen, or renal medulla. In 2 of the treated animals, focal hepatitis and hepatic necrosis were observed.

The *in vitro* aureomycin sensitivity of the original organism used, when grown in a modified Dubos liquid medium, was 100 μg/cc. The sensitivity of the strain recovered from the guinea pig after 6 weeks of aureomycin treatment was unchanged.

These results confirm the findings of Steenken and Wolinsky,[1] who failed to influence favorably guinea pig tuberculosis using a considerably lower dosage of aureomycin. It is shown here that aureomycin given in parenteral per kg dosage approximately 10 times the maximum used for other infections in man fails to influence favorably the course of guinea pig tuberculosis, and, indeed, exhibits definite toxicity.

[1] Steenken, W., Jr., and Wolinsky, E., *Am. Rev. Tuberc.*, 1949, **59**, 221.

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## "Crystalline" Virus-Like Particles from Skin Papillomas Characterized by Intranuclear Inclusion Bodies.* (17328)

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This is a report of the observation with the electron microscope of virus-like bodies that have been obtained from skin papillomas. These papillomas are characterized by the presence of intranuclear inclusion bodies, and the elementary bodies obtained from them tend to be arranged in a crystalline-like pat-

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† National Research Council Fellow in Medical Sciences.

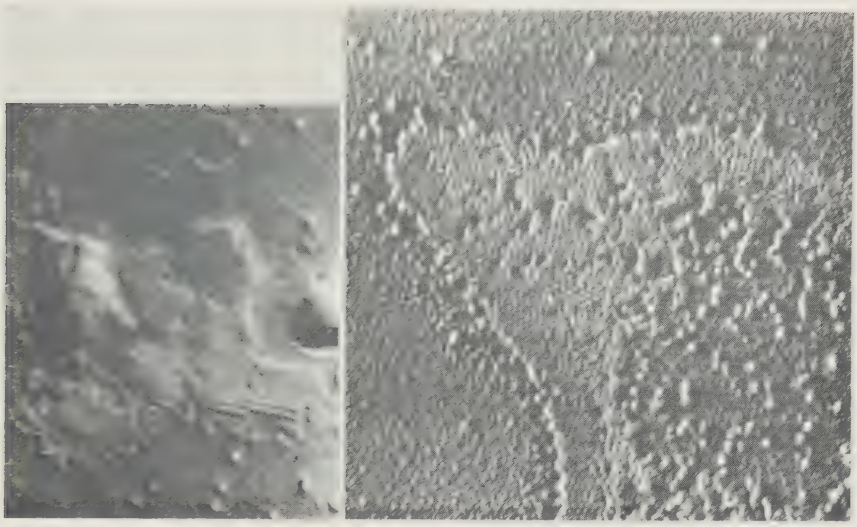

FIG. 1A. Electron micrograph of virus-like particles from intranuclear inclusion body papilloma. Note that particles are in crystalline-like array as well as free. Shadow cast with chromium, angle 1:7. Magnification 18,700 ×.

FIG. 1B. Same from another patient.

tern. Five such lesions were studied by electron microscopy. Four were obtained from the hands of children whose ages ranged from 2 to 12. These lesions differed from common warts in having a more pearly base and an erythematous halo, and were separated easily and completely from the underlying tissue with a curette. The fifth was a plantar wart obtained from a child 7 years old. It differed from the typical plantar wart in that there was no noteworthy hard hyperkeratotic top and very little surrounding callus. After a circular incision was made around the periphery this lesion also was easily and completely separated from the underlying tissue with a curette. Common warts from 9 patients, *molluscum contagiosum* lesions from 2 patients and one example of normal skin were similarly studied.

The preparation of the material was the same in all instances. Half of each lesion was fixed in formalin for tissue sections while the remainder was promptly ground with alundum and distilled water and subjected to clarifying centrifugation of the supernatant fluid at 2,000 r.p.m. for 5 minutes followed by centrifugation of the resulting supernatant fluid at 6,000 r.p.m. for 15 to 45 minutes. Small drops of the 6,000 r.p.m. supernatant fluid and the resuspended sediment were placed on collodion mounts for electron microscopy. These were shadow cast with chromium at an angle of 1:7.

Examination with the electron microscope (RCA type EMU) revealed spherical particles most abundantly in the 6,000 r.p.m. sediment of the suspension from the papillomas showing intranuclear inclusion bodies. These particles were frequently arranged in crystalline-like clusters or layers with an average diameter of 52 mμ and a range of 50 to 53 mμ (Fig. 1). Such an arrangement resembles that previously noted for crystalline plant viruses (Price, Williams, Wyckoff[1]). When these particles were not in crystal-like array, they averaged 68 mμ in diameter with a range of 56 to 80 mμ.

Control preparations of the *molluscum contagiosum* lesions revealed the characteristic brick-shaped elementary bodies previously noted by Boswell[2] (Fig. 2). These had an average length of 330 mμ with a range of 190 to 250 mμ. They were found most abundantly in the 6,000 r.p.m. sediment.

1 Price, W. C., Williams, R. C., Wyckoff, R. W. G., *Science*, 1945, **102**, 277.

2 Boswell, F. W., *Brit. J. Exp. Path.*, 1947, **128**, 253.

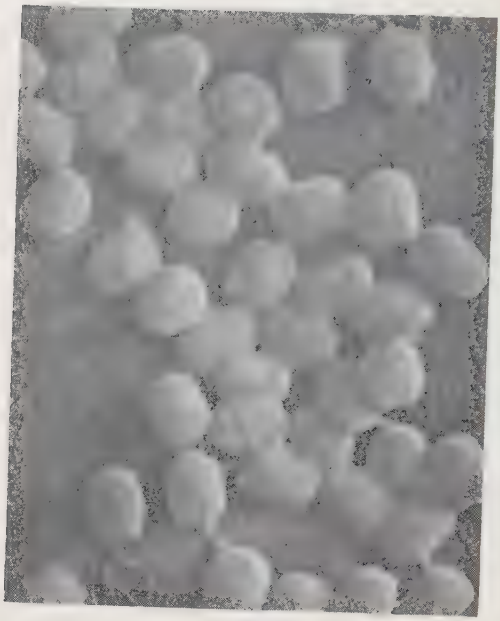

FIG. 2.
Electron micrograph of brick-shaped elementary bodies from molluscum contagiosum lesions. Magnification 35,000 ×.

These were also seen with the electron microscope in preparations made simply by touching the molluscum contagiosum lesions to the mount.

The common wart and normal skin preparations revealed no uniform particles, but merely amorphous scattered clumps of matter, collagen fibers, and spherical particles of varying diameter.

The histological appearance of the 5 papillomas yielding virus-like particles was similar (Fig. 3, 4, 5, 6). They showed irregular upgrowths of thickened epidermis overlying elongated papillae containing fine blood ves-

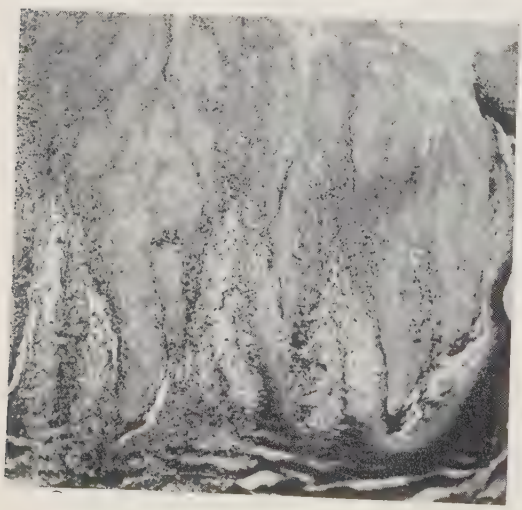

FIG. 3.
Papilloma yielding virus-like particles as shown in Fig. 1. Base is at bottom. Hematoxylin-eosin. Magnification × 30.

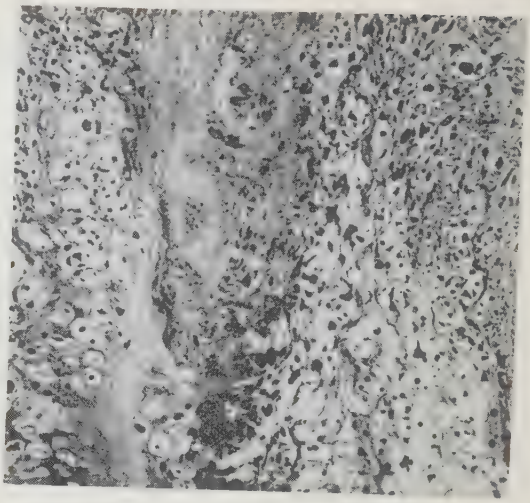

FIG. 4.
Higher power of a portion of Fig. 3—showing the prominent cytoplasmic bodies and looseness of lower cornified layers. Hematoxylin-eosin. Magnification × 65.

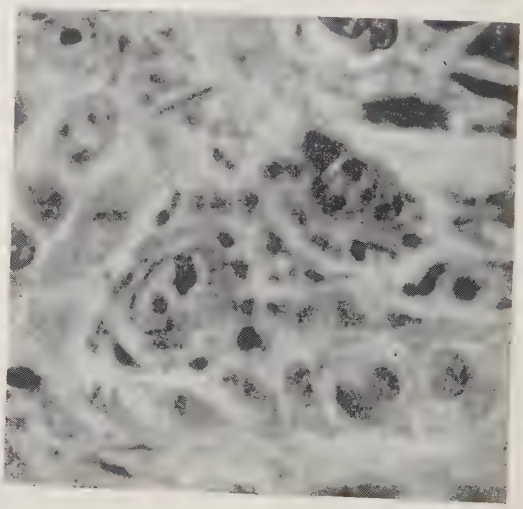

FIG. 5.
Same as Fig. 3. Note the single round intranuclear inclusion bodies photographing less black than the adjacent irregular chromatin masses. Solid cytoplasmic bodies are evident. Hematoxylin-eosin. Magnification × 1200.

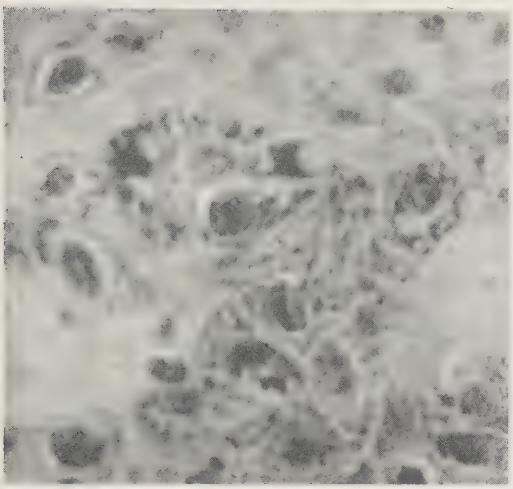

FIG. 6.
Same as Fig. 3. Both solid and vesicular cytoplasmic masses are conspicuous. Intranuclear inclusion bodies are also present. Hematoxylin-eosin. Magnification × 600.

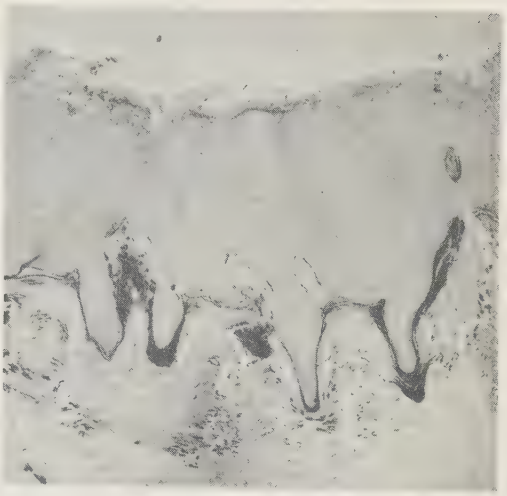

FIG. 7.
Common wart. Base is at bottom. Hematoxylin-eosin. Magnification × 30.

sels and delicate connective tissue. There was a great increase in the thickness of the stratum corneum with persistence of pyknotic nuclei in the lower layers where the keratinized substance was loosely arranged and not as compact as at the surface. Significantly, intranuclear inclusion bodies were present in a large proportion of the cells of the papillomas, but not of the adjacent epidermis. These were first seen in the layer directly over the basal-cells, and were evident in all of the more superficial layers. The inclusion bodies were round, stained with eosin and not hematoxylin; they were Feulgen negative and demonstrated little or no basophilia with methylene blue. Granules were present in the cytoplasm of the lowest row of cells that contained inclusion bodies. These granules when small were solid, but acquired a vacuole as they increased in size finally becoming oval or pointed in shape. Both the granules and the vesicular bodies stained with hematoxylin but had almost no affinity for the basic dye methylene blue and were Feulgen negative. At the level of the stratum granulosum they apparently became transformed into large solidly staining cytoplasmic bodies that persisted into the stratum corneum. Their affinity for hematoxylin was gradually replaced by a strong eosinophilia by the time they had reached the stratum corneum in the region showing the parakeratosis. No elementary bodies were seen in any of the stages in Giemsa preparations.

The lesions of *molluscum contagiosum* were classical: a localized zone of hyperplasia of the epidermis with eosinophilic bodies filling the cytoplasm, displacing the nuclei of the cells of the upper Malpighian layers and persisting into the stratum corneum where they eventually became hyaline and fragmented. The molluscum bodies were Feulgen positive. The common warts had the usual histological appearance (Fig. 7): papillomas composed of hyperplastic epidermis covering prolonged papillary processes bearing vessels and a delicate stroma. Considerable hyperkeratosis with variable parakeratosis was present.

Search through the accumulated material of the laboratory of surgical pathology of the New Haven Hospital revealed 31 other skin lesions histologically similar to that of the inclusion body papillomas described above. The ages of these patients ranged from 2½ to 68 years. There were also 158 typical verrucae from individuals 5 to 80 years of age.

*Summary.* Virus-like particles that tend to be arranged in a crystalline-like pattern have been observed by electron microscopy in a

TABLE I.

| Clinical diagnosis | No. | Electron microscopy: Spherical particles | Electron microscopy: Spherical particles in crystalline-like array | Electron microscopy: Brick-shaped bodies | Pathology: Intranuclear inclusion bodies | Pathology: Molluscum bodies |
|---|---|---|---|---|---|---|
| Intranuclear inclusion body papilloma | 5 | 5 | 4 | — | 5 | — |
| Molluscum contagiosum | 2 | — | — | 2 | — | 2 |
| Common warts | 9 | — | — | — | — | — |
| Normal skin | 1 | — | — | — | — | — |

study of suspensions of 5 skin papillomas. Histological examination of the same tissues revealed intranuclear inclusion bodies and characteristic cytoplasmic masses in the cells of the hyperplastic epidermis. Thirty-one lesions with similar histological appearance were encountered along with 158 typical verrucae in a review of surgical pathological material.

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## Studies on the Thromboplastic Agent in Plasma. (17329)

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Thromboplastic preparations can conveniently be divided into two categories, those containing proteins and those which are lipids free from protein. In a recent study on the mode of action of Dicumarol[1] evidence was presented indicating that the thromboplastic agent in plasma responsible for the clotting of recalcified plasma had the physiological characteristics of a lipid thromboplastic agent. Further evidence has been obtained consistent with this view and will be presented in this paper. This evidence depends on a demonstration of a direct proportionality between the coagulation time obtained with the thromboplastic lipid and the coagulation time of a control sample of the same plasma which was recalcified and to which no thromboplastic agent was added. Such a relationship can be explained most simply on the basis that the thromboplastic agent in plasma has the characteristics of the lipid thromboplastic agent. Such a relationship also implies, as will be discussed later, a mutual dependence on some limiting factor found in plasma.

*Methods.* The thromboplastic lipid was prepared from beef brain as outlined in the previous study.[2] It was stabilized by adding hydroquinone to make up 1% of its weight. This substance is an antioxidant that prevents the autoxidation of the preparation and its subsequent loss of activity.[3] In assaying the thromboplastic lipid a 0.2% emulsion in 0.9% NaCl was prepared from the dry material. The coagulation times of recalcified dog plasma were determined at room temperature with and without the thromboplastic lipid on 22 different occasions extending over a four month period. The plasma was obtained by withdrawing 9 parts of blood from the femoral artery of an unanaesthetized dog

[1] Lein, J., and Lein, P. S., *Am. J. Physiol.*, 1948, **155**, 394.

[2] Hays, H. W., and Lein, J., *Arch. Biochem.*, 1945, **7**, 69.

[3] Lein, J., and Lein, P. S., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 446

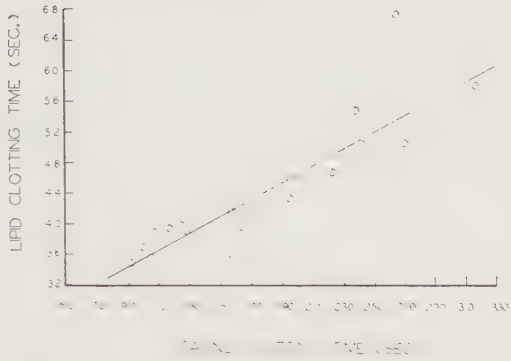


FIG. 1.
Linear relationship between clotting times obtained with and without the addition of the thromboplastic lipid to various samples of plasma.

directly into 1 part of 0.13 M sodium citrate. The blood was centrifuged for 30 minutes at 2,500 r.p.m. and the upper three-fourths of the plasma used. The clotting mixture consisted of 0.6 cc plasma, 0.2 cc of 1% $CaCl_2$, and 0.2 cc of either 0.9% NaCl or of 0.2% thromboplastic lipid emulsion in 0.9% NaCl. The $CaCl_2$ solution was always added last and the coagulation time determined with a stop watch from the time of mixing of the clotting mixture to the time the tube could be inverted with no flow of its contents. The tubes used were small test tubes 10 x 75 mm. Two determinations were done in each case and the average used providing the average deviation did not exceed 10% of the mean. In a few cases where the deviations were greater, additional determinations were carried out.

*Results.* There was a considerable variation in the coagulation times of different samples of plasma. This was true in those determinations in which the thromboplastic agent was added as well as those in which only saline was added. The variation, however, was not random. The results indicate a definite relationship between the clotting time obtained without the addition of the thromboplastic lipid and that obtained with its addition. The results are presented graphically in Fig. 1, each of the points representing determinations on a different sample of plasma. The points were fitted by a straight line using the method of least squares. The significance of the regression coefficient was tested statistically by the null hypothesis[4] and was found to be highly significant (df = 20, t = 12.3).

*Discussion.* The shortening of coagulation time by the addition of thromboplastic agents coupled with the fact that there is a great excess of prothrombin[5] and of fibrinogen[6] in normal plasma makes it evident that a limiting factor in coagulation when calcium ions are added in optimal quantities is the amount of thromboplastic agent present. The results have indicated that a linear relationship exists between the accelerated clotting time caused by the addition of the thromboplastic lipid and the clotting time of the plasma without this addition. The possibility that this dependence is due merely to the additive effect of 2 thromboplastic substances is excluded since the concentration of the lipid used was in excess of the amount necessary to produce the maximum shortening of coagulation time.[2] With this possibility ruled out, it becomes difficult to explain the results in terms of the classical ideas of thromboplastin action. If the lipid thromboplastic agent reacts with prothrombin in the presence of calcium ions to form thrombin, one would not expect there to be such a dependence as was demonstrated.

The results are believed to be explained most simply by the hypothesis that both the thromboplastic agent in the plasma and the thromboplastic lipid have the same mechanism of action and that the clotting of plasma by both of these agents depends on some limiting factor found in the plasma. The latter assumption is necessary since the results are obtained with optimal quantities of the thromboplastic lipid. The variation of clotting times of the different plasma samples is thought to be due to the variation in the plasma factor since prothrombin is present in more than adequate concentrations. According to this view the lipid thromboplastic agent prepared from tissues requires another factor found in plasma in order to become active. This is not true for protein thromboplastic agents since the work of Quick[7] and

[4] Snedecor, G. W., Statistical Methods, Iowa State College Press, Ames, 3rd ed., 118.

[5] Tanturi, C. A., and Banfi, R. F., *J. Lab. and Clin. Med.*, 1946, **31**, 703.

[6] Witts, L. J., *J. Path. and Bact.*, 1942, **54**, 516.

[7] Quick, A. J., *Science*, 1940, **92**, 113.

others showed that the plasma clotting times with these agents are absolute and depend only on the prothrombin concentration when it becomes limiting.

*Summary.* Statistical evidence is presented for a relationship between the coagulation time of recalcified plasma and the coagulation time accelerated by the addition of a thromboplastic lipid. This relationship is thought to imply that the thromboplastic substance in plasma responsible for the clotting of recalcified plasma has the same physiological characteristics as the lipid thromboplastic agent and that both require for activity some limiting factor present in plasma.

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## Effect on Prothrombin of Acute Massive Plasmapheresis with Simultaneous Chloroform Intoxication. (17330)

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From the earliest work on blood clotting, plasma prothrombin has been recognized as an integral component in the mechanism of thrombin formation. The effects on prothrombin of diverse pathologic and physiologic states have been studied extensively in the clinic and laboratory. Because prothrombin is so intimately related to liver function, an appreciable understanding of the origin, mode of action, and utilization of prothrombin may be acquired from subjecting the liver to various abnormal chemical and physical conditions. Experimental evidence and clinical experience with certain forms of liver and biliary tract disease indicate that plasma prothrombin is produced through the vital activity of the liver.[1-7] Thus experimentally induced hepatic necrosis,[1,2] and partial[3] or complete hepatectomy[4,5] result in hypoprothrombinemia.

Many factors, in addition to the fundamental integrity of the liver, are known to operate in the maintenance of plasma prothrombin. An adequate intake of Vitamin K is essential. Certain drugs, notably dicumarol, cause hypoprothrombinemia without any morphologic evidence of damage to liver tissue. Yet very little is known about the mechanism by which prothrombin is produced and utilized in the body.

There is considerable evidence to suggest that plasma prothrombin will gradually disappear over a period of 1-3 days if the production of new prothrombin is impaired. Thus the minimum prothrombin concentration is reached in 36-48 hours following severe chloroform poisoning,[1,2] or partial hepatectomy.[8] If animals are rendered hypoprothrombinemic as a result of Vitamin K deficiency,[8] or dicumarol poisoning,[9] transfusions with normal blood result in a temporary elevation of prothrombin concentration. The effect of transfusions lasts only 1-2 days. Purified bovine prothrombin administered intravenously lasts 2-3 days in a dog made hypoprothrombinemic with dicumarol.[10] On the other hand, when prothrombin production is impaired, the tendency of plasma prothrombin concentration

1 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

2 Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

3 Warner, E. D., *J. Exp. Med.*, 1938, **68**, 831.

4 Warren, R., and Rhoades, J. E., *Am. J. M. Sc.*, 1939, **198**, 193.

5 Andrus, W. D., Lord, J. W., and Moore, R. A., *Surgery*, 1939, **6**, 899.

6 Bollman, J. L., Butt, H. R., and Snell, A. M., *J.A.M.A.*, 1940, **115**, 1087.

7 Brinkhous, K. M., *Medicine*, 1940, **19**, 329.

8 Smith, H. P., Warner, E. D., Brinkhous, K. M., and Seegers, W. H., *J. Exp. Med.*, 1938, **67**, 911.

9 Quick, A. J., The Hemorrhagic Diseases, Charles C. Thomas, 1942, p. 283.

10 McGinty, D. A., Seegers, W. H., Pfeiffer, C. C., and Loew, E. R., *Science*, 1942, **96**, 540.

to stabilize at a normal level, since liver tissue continues to regenerate, suggests a decrease in the rate of prothrombin utilization. This, with some type of liver damage, with concomitant abnormal accumulation of bile constituents but inadequate failure of the prothrombin concentration tends to reach an equilibrium. Were the utilization rate constant, it would seem that the decline resulting from inadequate production would continue. Thus the rapid fall of prothrombin concentration, followed by relatively stable but subnormal levels over many days, may be more rapid than allowed by experimental liver damage.

The purpose of the experiments reported in this paper was to determine the effect of massive plasmapheresis on the disappearance rate of prothrombin from the blood stream after severe chemical intoxication, and to correlate the changes in prothrombin concentration with those of the total protein constituents of the plasma in dogs.

The data resulting from the present experiments entirely confirm previous findings[1] relative to the essential role of the liver in the maintenance of prothrombin. In addition, the experiments indicate that 1) no significant reserve source of prothrombin exists, 2) when the prothrombin production rate is decreased, there is a concomitant decrease in the rate of its utilization, and 3) following chemical intoxication the impairment of the prothrombin producing potential of the liver develops gradually over a period of 3–4 days.

Dietary diets ranged from 5.3–12.5 kg. were used in all experiments. No attempt was made to control the diets rigidly, nor was any particular relation between the size and experimental observations noted. The low protein, high carbohydrate diet consisted of boiled whole wheat, cooked cereal, boiled potatoes, with very little meat. The meat diet was composed of boiled whole wheat supplemented with pork, lean meat. The high protein diet consisted of meat and milk powder only. Tissue blood was obtained from dogs maintained on a meat diet. Following removal of the plasma by plasmapheresis, the cells were washed twice with and resuspended in Locke's solution. Sodium

TABLE I

| Dog | 401 | 403 | 403 | 404 | 405 | 406 | 408 | 409 | 410 | 411 | 412 | 413 | 414 | 415 | 416 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type of experiment | Plasmapheresis | Plasmapheresis | Control | Control | Control | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis | Plasmapheresis |
| Hours of fasting before CCl4 and plasmapheresis | 0 | 30 | 30 | 30 | 0 | 30 | 60 | 30 | 30 | 30 | 0 | 30 | 30 | 30 | 30 |
| % reinjected blood volume removed by plasmapheresis | 100 | 65 | - | - | 60 | 58 | 84 | 93 | 93 | 87 | 87 | 87 | 58 | 105 | 89 |
| % plasma proteins removed by plasmapheresis | 50 | 86 | | | 83 | 81 | 84 | 83 | 84 | 87 | 83 | 83 | 45 | | |
| % prothrombin removed by plasmapheresis | 60 | 52 | | | 51 | 55 | 48 | 58 | 84 | 58 | 58 | 71 | 86 | 84 | |
| Condition of dog after plasmapheresis and CCl4 | Dead, shock | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma | Dead, coma |
| Complete recovery of prothrombin (days) | | 18 | 8 | 8 | 8 | 1 | 10 | 8 | 6 | 8 | 8 | 8 | 2 | 2 | 6 |

P = Plasmapheresis; C = Chloroform

loid substances were supplemented. A period of starvation preceded the chloroform anesthesia. (Table I.) Anesthesia was induced first with ether. The dogs subjected to chloroform and plasmapheresis were given deep chloroform anesthesia for from 75-100 minutes. Under ether anesthesia, immediately following chloroform, the femoral vessels were exposed and cannulated. The bottle containing warmed donor blood cell suspension was attached to an air pressure system so that the transfused blood entered the vein at virtually the same rate as blood was removed from the artery. Throughout the actual exchange, equal volumes of recipient and donor blood were constantly maintained. The rate of exchange was not allowed to exceed 100 cc per minute. The fraction of the calculated blood volume removed is indicated in per cent in Table I. The calculated blood volume was based on 90 cc per kg of body weight. Naturally, the blood removed during the pheresis procedure represents an admixture of the recipient's own blood and donor cells. The percentage of prothrombin and plasma proteins removed by the pheresis procedure was determined by calculation of differences of the levels before and immediately after plasmapheresis. Blood for the analyses was drawn from the jugular vein into a syringe wet with oxalate solution, and gently expressed into an 8 cc hematocrit tube containing 1.85% potassium oxalate. Prothrombin determinations were done by the two-stage method of Warner and Smith.[1,2] Crude lung extract which is rather rich in accelerator factor(s) was used as the source of thromboplastin. Protein values were determined by the semi-micro Kjeldahl method using the Parnas-Wagner distillation apparatus.

*Experimental Observations.* Prothrombin, total plasma protein, and hematocrit values in a typical experimental dog (45-4a) following prolonged chloroform anesthesia are recorded graphically in Fig. 1. The fall of prothrombin is extreme, the lowest level occurring at 40-48 hours. It is at this time that the icteric index is maximal, although the first appearance of icteric plasma is usually noted several hours earlier. Depending upon the depth and length of chloroform anesthesia, and the degree of relative anoxia, the prothrombin concentration at the end of 48 hours may be very low. There is a concomitant elevation of hematocrit values indicating moderate hemoconcentration. Prothrombin recovery begins promptly on the third day and is complete by the eighth day. This recovery coincides with liver repair which begins in about 48 hours and is almost complete in 5-6 days. It is noteworthy that the plasma protein values fall moderately during the period of maximal liver regeneration and prothrombin recovery. This phenomenon was repeatedly observed, and its possible significance will be discussed later.

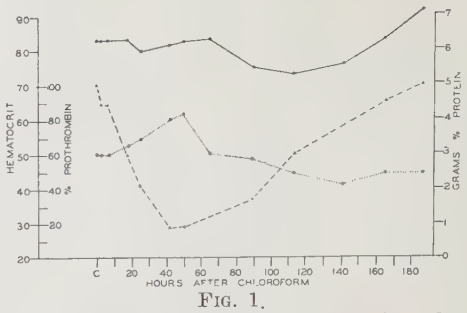


FIG. 1.

Effect of chloroform on prothrombin, plasma protein, and hematocrit values.

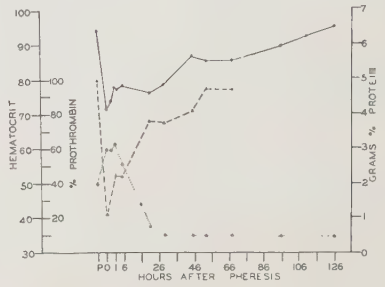


FIG. 2.

Effect of plasmapheresis on prothrombin, plasma protein, and hematocrit values.

Fig. 2 shows the prothrombin, total plasma protein, and hematocrit values of a typical experimental dog (45-10b) following acute massive plasmapheresis. The precipitous fall of prothrombin and plasma proteins is roughly proportional to the amount of blood removed. At such low prothrombin levels, bleeding from venapuncture wounds frequently occurred; no spontaneous bleeding was observed, however. The recovery curve of prothrombin follows a rather characteristic

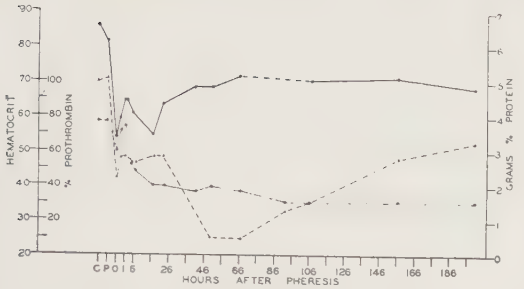


FIG. 3.

Effect of chloroform and plasmapheresis on prothrombin, plasma protein, and hematocrit values.

pattern, with a return to about 75% of normal in from 16 to 20 hours following plasmapheresis. By the end of 50 hours, prothrombin values have returned to normal. The recovery rate of the total proteins is more prolonged. It will be noted that after an abrupt rise of approximately 20% above the minimum value, the plasma proteins remain relatively constant for a period of about 26 hours, while during the same period, the prothrombin recovery rate is maximal. From 26-46 hours, the recovery rates of both prothrombin and plasma proteins are virtually the same, although for the latter, this period marks the maximal recovery rate. At the end of 116 hours, the plasma protein level has returned to normal. The sharp rise followed by the abrupt fall of the hematocrit is adequately explained by hemoconcentration followed by relative anemia.

When dogs are subjected to both acute massive plasmapheresis and chloroform intoxication, the graphical result is similar in several respects to a superimposition of the chloroform curve upon the pheresis curve. There are certain significant differences, however. Fig. 3 shows the results of a typical experiment of this type (dog 45-4b). The fall of prothrombin concentration is abrupt following plasmapheresis. This is followed by an exceedingly rapid increase in prothrombin values, probably due to hemoconcentration initially. The prothrombin concentration is maintained at this elevated level for a period of approximately 20 hours. This is in sharp contrast to the very transitory elevation of plasma protein and hematocrit values. Numerous attempts were made to alter the character of this segment of the prothrombin curve, but regardless of diet, the quantity of exchanged blood, or the degree of chloroform intoxication, a postpheresis plateau invariably was obtained. At about 26 hours, prothrombin values again begin to fall and continue to do so for an additional 20 hours. If one graphically compares the decrease in prothrombin concentration after 26 hours with that of the comparable period in dogs subjected to chloroform intoxication only, it is observed that the curves are similar. This indicates that the termination of the postpheresis plateau and the subsequent decline in prothrombin concentration is the result of liver damage initiated some 30 hours previously by chloroform. As in the case of chloroform alone, the minimum prothrombin concentration is reached by the end of 48 hours. This is followed by a slow, but progressive recovery period.

The first segment of the plasma protein curve (Fig. 3) follows the pattern of the prothrombin curve rather closely. The rapid fall of plasma proteins parallels the fall of prothrombin. In every instance, the edema level was reached or exceeded, but, probably because this was only transitory, no clinical edema was noted. The postpheresis recovery curves of plasma protein values were quite variable, but generally speaking, there was a transitory rise with a secondary fall comparable to that of the hematocrit values. In the experiment shown in Fig. 3, concomitant with the fall of prothrombin, incident to the hepatotoxic effect of chloroform, plasma protein values gradually rose from 3.5 g% to 5.2 g%. This level was maintained for approximately 80 hours, during which time prothrombin recovery was most rapid. The general pattern of the plasma protein curve showed considerable variation in different animals, however. In some animals, there was a definite but minimal fall of plasma proteins during the period of maximal liver injury. Often, an additional drop in plasma proteins was observed during maximal prothrombin recovery. In other animals, plasma protein recovery paralleled prothrombin recovery. The

hematocrit values frequently showed a slight decrease immediately following plasmapheresis. A transitory rise, presumably due to hemoconcentration, then occurred in all of the dogs. This rise lasted only one to 2 hours in contrast to the postpheresis rise in prothrombin values, however. Considerable hemolysis was unavoidable in the massive pheresis procedure so that even though the hematocrit values of donor cell suspension and recipient blood were virtually the same, many of the washed red blood cells were hemolyzed. The result was a moderate anemia. The hematocrit values leveled off at from 30 to 40% and gradually returned to normal after 2-3 weeks.

*Discussion.* The profound fall in plasma prothrombin concentration in dogs having been subjected to chloroform intoxication confirms the previous work of Warner, Smith, and Brinkhous.[1,2] The evidence indicates that the liver is the source of prothrombin production, since the fall of prothrombin concentration rather closely parallels the degree of liver injury, and the prothrombin returns to normal as the liver regenerates.

Kerr, Hurwitz, and Whipple,[11] showed that severe liver injury in dogs by means of phosphorous or chloroform intoxication will cause, or at least be associated with, a moderate fall in blood plasma proteins. With 1½ hours of chloroform anesthesia, the fall began on the second day and progressed for the next 3 or 4 days during which period liver repair was most active. With comparable liver injury, our data (Fig. 1) revealed a similar plasma protein response, the minimal level occurring at about 4½ days. For almost 3 days following the administration of chloroform, during which period the prothrombin concentration had reached its lowest level and had begun to return toward normal values, the plasma proteins had remained relatively stable at a normal level. This lag period, occurring concomitantly with that of the greatest liver injury, could be explained by the presence of reserve stores of plasma proteins, if one assumes that the liver is the source of albumin and globulin and that the rate of utilization remains constant at a normal level. If this assumption is valid, there must be some decrease, if not a total arrest, in the rate of plasma protein production resulting from liver injury. The lag period might also be taken as evidence, although inconclusive, that tissues other than liver fabricate albumin and globulin. Nevertheless, the extensive work of Whipple, Madden *et al.* strongly favors the liver as the source of the plasma proteins.[12] There are at least two other possible explanations for the decline of plasma proteins during the period of liver repair and maximal prothrombin regeneration. A smooth line curve of the plotted data accentuates the fact that the decline of plasma proteins coincides with an increase in prothrombin concentration, thereby suggesting a relationship between the two. Although prothrombin is only a very small fraction of the total proteins, it is conceivable that in order for prothrombin to be fabricated rapidly following liver injury, relatively large amounts of plasma proteins are diverted from the circulation to provide a pool from which the prothrombin molecule is constructed. Or, it is possible that the circulating proteins are called upon to supply the amino acid moiety necessary to fabricate liver protein itself, since the fall in plasma proteins occurs during the period of regeneration of liver tissue.

The rapid removal of as much as 50% of the circulating prothrombin results in the expected abrupt reduction of the level in the blood. On the basis of evidence cited above, that prothrombin in the blood lasts 1-3 days,[1-5,8-10] the removal of such amounts of prothrombin in animals subjected to chloroform intoxication would be expected to result in a conspicuously lower prothrombin level throughout the remainder of the experimental period. On the contrary, plasmapheresis does not appear to increase the severity of the hypoprothrombinemia, nor does it appear to alter the rate of prothrombin regeneration during the recovery period. The postpheresis plateau in chloroformed dogs could be explained on

[11] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918, **47**, 379.

[12] Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

the basis of available prothrombin from reserve stores. Were this the case, however, once the reserves were exhausted, the prothrombin concentration would be expected to fall rapidly to a point below that of the chloroformed animals not subjected to plasmapheresis. Also, the rate of disappearance of prothrombin following total hepatectomy suggests that no significant stores exist. Further, with massive plasmapheresis in the normal animal, the fall in prothrombin concentration approximates the expected decrease from the amount of blood exchanged, and the rate of recovery is comparable to that seen in chronic Vitamin K deficiency following administration of specific therapy. Were appreciable quantities of prothrombin present in stores, it would be expected that the fall would be less and the recovery rate more rapid in the acute pheresis animal.

The relative ineffectiveness of massive plasmapheresis to materially alter the prothrombin level beyond 24-36 hours, despite greatly impaired liver function, could be explained on the basis of a very rapid turnover of the plasma prothrombin. Thus, if under normal conditions, the prothrombin of the blood was replaced several times per hour, the amount removed by plasmapheresis would become relatively insignificant. However, such rapidity of turnover does not seem compatible with the rate of fall of prothrombin following total hepatectomy.[4,5]

Evidence now available suggests that for any given level of hepatic function relative to prothrombin production, a balance is established between the rate of utilization and rate of formation. As a result, the plasma prothrombin becomes stabilized at a subnormal level, the height of which is determined more by productive capacity, than by any semi-constant rate of utilization. This is in accord with the tendency for the plasma prothrombin to become stabilized at a subnormal level when animals are maintained on a low Vitamin K or continuous dicumarol intake.

Our observations would strongly indicate, therefore, that no significant reserve stores of prothrombin exist, but rather that a nice balance between prothrombin production and utilization best explains the experimental findings. Indeed, even the postpheresis plateau is probably an artefact, since if we compare the fluctuation of plasma protein and prothrombin values with those of the hematocrit, it will be seen that fluctuations of plasma volume might well result in an apparent increase per unit volume of plasma proteins and prothrombin.

Calculations involving total grams of plasma proteins and total units of prothrombin, respectively, 1) before plasmapheresis, 2) actually removed, and 3) remaining in the circulation, 1-10 minutes after pheresis, were made. The respective values were corrected for oxalate and hematocrit. The results were in accord with the fact that reserve stores of plasma protein exist, but indicated that no significant, if any, reserve stores of prothrombin are present. Since no total plasma volume determinations were performed, however, the calculations are inconclusive. Wide fluctuations in total plasma volume could readily distort the total values of prothrombin and plasma proteins.

From the experimental data, it can also be concluded that hepatotoxic damage with the concomitant decrease in prothrombin concentration incident to chloroform intoxication, is a progressive phenomenon which develops gradually over a period of 1-2 days. If the liver were completely functionless after the first hour of chloroform, the postpheresis plateau would then be impossible. Rather the prothrombin would continue to fall at a rate determined by utilization alone.

*Summary.* Dogs subjected to both chloroform intoxication and acute massive plasmapheresis reveal changes in prothrombin concentration which rather conclusively indicate that no significant reserve stores of prothrombin exist. The stabilization of prothrombin at subnormal levels, as well as the rate of recovery of prothrombin, represents, instead, a balanced equilibrium between production and utilization. The data also substantiate the impression that hepatotoxic injury, with a concomitant decrease in prothrombin concentration, is a gradual progressive phenomenon.

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# Electrocardiographic Changes Produced on the Syrian Hamster (*Cricetus auratus*) by Diphtherial Toxins. (17331)

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The golden hamster, *Cricetus auratus*, is sensitive to diphtherial toxin and exhibits neurotoxic symptoms when toxins exposed to alkaline reactions are administered, whereas toxins kept at slightly acid or neutral reactions do not exert apparent effects on its nervous system.[1,2] Since the clinical syndrome of diphtheria is characterized by neurological disturbances as well as abnormalities in the cardiac function, it was decided to study the effect of diphtherial toxins on the heart of the golden hamster.

*Methods.* Toxin samples were prepared from filtrates of 8 day cultures of *Corynebacterium diphtheriae* (Park No. 8 strain). With the aid of highly diluted HCl or NaOH the toxins were brought to the proper pH level and kept at it for 8 days at 30°C as described elsewhere.[1,2] Clinical and electrocardiographic examinations were made on a total of 24 hamsters; special attention was paid to the appearance of peripheral paralysis. Limb leads electrocardiograms were taken on a portable amplifier instrument. Small (0.5 : 1.2 cm) electrodes of German silver were applied after the skin had been rubbed with electrode jelly.

*Results.* In 5 apparently normal hamsters electrocardiograms were taken at 3 day intervals for one month. They showed within this period a constant pattern, the various deflections being similar to those of the human electrocardiogram. The P wave was indistinct owing to the rapid heart rate, 300-400 per minute, as shown in Fig. 1, left side.

Ten hamsters received minimal lethal doses of a toxin kept at pH 6.7; their average survival time was 8 days. No neurotoxic symptoms appeared; daily electrocardiograms did not reveal any disturbance of the heart function. In 4 animals we succeeded in obtaining electrocardiograms 4 hours before death; they were found to be normal. At gross pathological examination the classical signs of diphtherial intoxication were found: edema and necrosis at the site of injection and hemorrhages in the adrenal glands.

Another group of 11 hamsters received minimal lethal doses of a toxin exposed to pH 7.3. In 5 of them paresis appeared and their electrocardiograms showed marked abnormalities, summarized in the upper part of Table I. These changes preceded by some days the appearance of neurological abnormalities. In 6 other animals the electrocardiogram and the neurological functions remained normal.

A third group of 8 animals received minimal lethal doses of a toxin exposed to pH 8.5; their average survival time was 21 days. All of them showed paresis and marked electrocardiographic disturbances, summarized in the second part of Table I. (*cf.* Fig. 1, right).

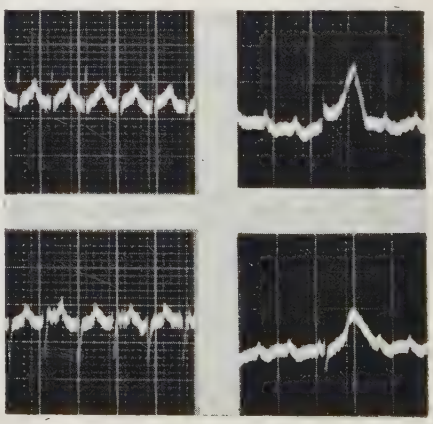

Fig. 1.
Left: Electrocardiogram (leads II and III) in a normal hamster.
Right: Auriculo-ventricular block after the injection of a diphtherial toxin kept at pH 8.5.

[1] Olitzki, L., Stuczynski, L. A., and Grossowicz, N., *4th Internat. Cong. f. Microbiol. Copenhagen, 1947; Rep. Proc.*, 1949, p. 180.

[2] Olitzki, L., Stuczynski, L. A., and Grossowicz, N., *J. Immunol.*, 1948, **60**, 419.

TABLE I.
Paresis and Heart Disturbances Produced in Hamsters by Minimal Lethal Doses of Diphtherial Toxins Exposed to Alkaline Reactions.

| No. hamster | Toxin kept at pH | First appearance (days) of disturbances of the | | Survival, days | Functional abnormalities observed |
|---|---|---|---|---|---|
| | | Nervous system | Heart function | | |
| 1 | 7.3 | 4 | 3 | 5 | Ectopic beats |
| 2 | 7.3 | 8 | 6 | 9 | " " |
| 3 | 7.3 | 10 | 7 | 25 | " " |
| 4 | 7.3 | 5 | 4 | 6 | Partial auriculo-ventricular block |
| 5 | 7.3 | 11 | 7 | 12 | Ectopic beats |
| 6 | 8.5 | 23 | 23 | 30 | " " , later total auriculo-ventricular block |
| 7 | 8.5 | 25 | 25 | 28 | Like No. 6 |
| 8 | 8.5 | 7 | 7 | 9 | total auriculo-ventricular block |
| 9 | 8.5 | 7 | 7 | 12 | Like No. 8 |
| 10 | 8.5 | 22 | 22 | 28 | Like No. 8 |
| 11 | 8.5 | 22 | 22 | 30 | Ectopic beats |
| 12 | 8.5 | 8 | 8 | 18 | Partial auriculo--ventricular block |
| 13 | 8.5 | 8 | 8 | 14 | Like No. 12 |

TABLE II.
Paresis and Heart Disturbances Produced in Hamsters by Sublethal Doses of Diphtherial Toxins Exposed to Alkaline Reactions.

| No. hamster | Toxin kept at pH | Paresis, day of | | Ectopic beats, day of | |
|---|---|---|---|---|---|
| | | First appearance | Recovery | First appearance | Recovery |
| 1 | 7.3 | 16 | 46 | 9 | 90 |
| 2 | 8.5 | 10 | 44 | 20 | 150 |
| 3 | 9.0 | 17 | 35 | 22 | 150 |
| 4 | 9.0 | 17 | 45 | 22 | 270 |

In a group of 4 hamsters the action of sublethal doses (0.2-0.5 MLD) of toxins was examined. In this group the neurotoxic symptoms were of a transient character and disappeared about 3 to 4 weeks after their first appearance. The electrocardiographic abnormalities, on the other hand, persisted much longer and in one hamster were apparent 9 months after the injection of the toxin. (Table II).

*Summary and conclusions.* Diphtherial toxins exposed to alkaline reactions produced in the golden hamster, in addition to neurotoxic symptoms, marked disturbances in the heart function as indicated by electrocardiographic abnormalities. Acid-treated toxins on the other hand, did not cause any electrocardiographic changes. The first appearance of the electrocardiographic changes frequently preceded that of the paralytic symptoms, and when sublethal doses were administered, the electrocardiogram remained abnormal even after the neurological symptoms had disappeared. Electrocardiographic examination of the heart function seems, therefore, to be a more sensitive method of measuring the action of certain diphtherial toxins than the observation of the neurotoxic symptoms.

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# Distribution of Certain Enzymes in the Brain of the Pigeon.* (17332)

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In the course of investigations dealing with the functional significance of cerebral capillary patterns, a relationship was found between the vascularity of certain neuropils and the number of mitochondria present.[1] The relationship was considered significant because mitochondria are carriers of enzymes.[2–4] This fact and the availability of histochemical methods for the demonstration of enzymes in tissues suggested a study of the distribution of enzymes in the brain as one aspect of the relationship between cerebral vascularization and brain metabolism.

*Materials and methods.*† Pigeon brains were used in this study because it was found from a survey of the brains of various vertebrates that the histochemical methods available for the demonstration of enzyme activities gave the best over all results in pigeon brains. The methods used were those of Gomori[5] and Manheimer and Seligman[6] for alkaline phosphatase, and those of Gomori for acid phosphatase,[7] phosphamidase,[8] and cholinesterase.[9] Although these methods represent valuable additions to the existing histochemical methods, caution is required in the interpretation of the results that may be obtained from their application. Several investigators[10–12] have shown that there is a definite unavoidable destruction and loss of the phosphomonoesterases, particularly acid phosphatase, as a result of the fixing, embedding, and mounting processes to which the tissues are subjected. In fact, the validity of the assumption that the results obtained by the acid phosphatase method are wholly or at all due to the activity of this particular enzyme has been seriously questioned.[11,13,14] The accuracy of the localization of alkaline phosphatase activity has been doubted,[15] but the sharp boundaries in our pictures indicate that there occurred little if any diffusion. In regard to the demonstration of cholinesterase activity Koelle and Friedenwald[16] suggest that the method of Gomori localizes only a non-specific enzyme and that there may be other undemonstrated cholinesterases present which have different substrate specificities.

In preparation for the enzyme determinations the tissues were fixed in chilled acetone or absolute alcohol, were transferred to benzene and were infiltrated with and embedded in paraffin in a vacuum oven. The sections were cut at 5$\mu$. The substrates used were sodium alpha glycerophosphate in the acid and alkaline phosphatase methods of Gomori,

* This paper reports research undertaken under contract with the Office of Naval Research. The conclusions contained in this report are those of the authors and do not necessarily reflect the views of the Office of Naval Research.

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† We are greatly indebted to Dr. G. Gomori, University of Chicago, for helping us solve technical problems that arose in the course of the investigation.

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calcium beta naphthol in the alkaline phosphatase method of Manheimer and Seligman, p-chloranilidophosphonic acid in the phosphamidase method, and palmitoyl choline chloride in preference to myristyl choline chloride in the cholinesterase method.

*Results.* The patterns of the different enzyme activities were consistent in the brains examined. Alkaline phosphatase was demonstrated in an apparently great quantity in the pigeon brain (Fig. 1) by both the methods of Gomori, and Manheimer and Seligman. This observation is noteworthy because Gomori[17] and Bourne[18] state that alkaline phosphatase is not found in brain tissue. On the other hand we could not demonstrate in the pigeon the great quantities of acid phosphatase which are reported by these workers to be present in the brains of other animals. However, the faint patterns which we did obtain were consistent. Although the results of this procedure are difficult to interpret, we have included them in our comparative analysis of the different enzyme patterns.

From observations restricted to a few clearly defined structures, we wish to point out some striking differences in the enzyme content of a few areas in the brain keeping in mind the questions raised in one of the preceding paragraphs concerning the validity of the methods employed.

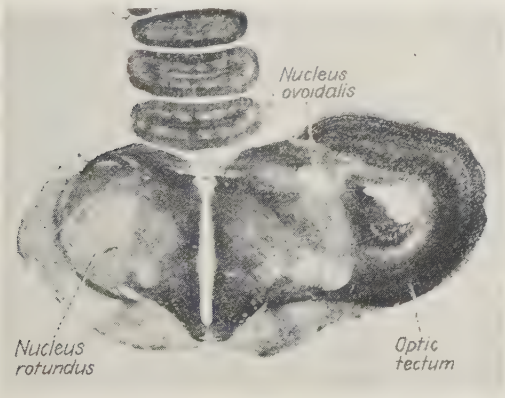


FIG. 1.

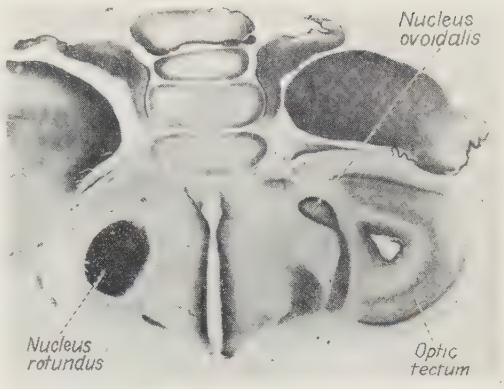


FIG. 2.

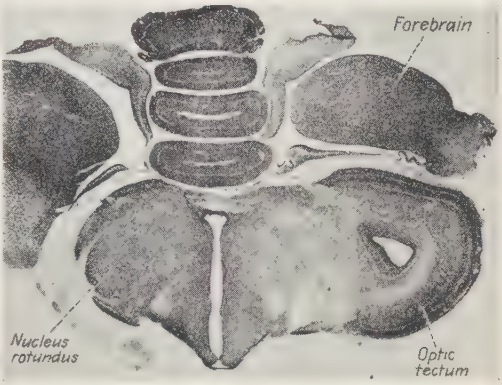


FIG. 3.

In adjacent sections the nucleus ovoidalis appears to be entirely lacking in alkaline phosphatase (Fig. 1), but seems very rich in cholinesterase (Fig. 2.). Its outline may be traced in a preparation which demonstrates acid phosphatase, but it can hardly be distinguished from other structures in a phosphamidase preparation (Fig. 3) inasmuch as its content of phosphamidase is about the same as that of the surrounding brain tissue.

The nucleus rotundus is similarly conspicuous in cholinesterase preparations (Fig. 2) whereas its outline can be barely identified in acid phosphatase preparations. Its content of alkaline phosphatase is markedly less than that of surrounding structures (Fig. 1); in phosphamidase preparations its boundaries are indistinct (Fig. 3).

The occipital pole of the forebrain shows some acid phosphatase and a high content of phosphamidase (Fig. 3) and of cholinesterase

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[18] Bourne, G., *Quart. J. Exp. Physiol.*, 1943, **32**, 1.

(Fig. 2); but it is entirely free of alkaline phosphatase (Fig. 1).

The observation of layers of enzymatic activity in the optic tectum (Figs. 1-3) is of particular interest in view of the corresponding arrangement of cells, fibers, blood vessels, and mitochondria in this part of the brain. A more detailed description of the distribution of enzymes in the optic tectum will be given in a separate paper.

*Discussion.* The statements "free of" or "rich in" as made in the description of our preparations should, of course, not be taken to mean absolute quantitative values, since there is, as has been pointed out above, an unavoidable loss of enzymatic activity that occurs in the process of fixing, embedding and mounting the tissues.

Consequently the results which we have obtained are only relative. For example the nucleus ovoidalis and the occipital pole of the forebrain appear in our preparations free of alkaline phosphatase. This may be only the result of the loss of the relatively small amounts of the enzyme which they contained in the living state. Other adjoining brain tissue may have had so much more alkaline phosphatase to begin with that even after having been subjected to the same technical procedures there is still enough of the enzyme left to give a marked reaction with the methods employed. The fact however remains that there are demonstrable differences in enzyme content.

The distribution of cholinesterase activity shown in our preparations is of interest in comparison with the results of Feldberg and Vogt.[19] These authors assayed very small amounts of tissue taken from 40 different regions of the central nervous system; they found pronounced differences in the content of the enzyme or enzyme system synthesizing acetylcholine in the different regions examined. The conclusion of Feldberg and Vogt that in the central nervous system cholinergic neurons alternate with noncholinergic ones suggests the possibility that our pictures of the distribution of cholinesterase in the pigeon brain may reflect the actual situation rather than inadequacies of the method.

It is evident that at present no conclusion should be drawn from these observations as to the differences in actual metabolic functions of various parts of the brain. But it is clear that such areas as the nucleus rotundus and the nucleus ovoidalis differ not only in anatomical structure but also in some way biochemically from the surrounding brain tissue. For instance processes which require an enzyme to catalyze the hydrolysis of phosphomonoesters within the pH range 9.0 to 9.4 evidently occur to a much smaller extent in the nucleus rotundus and nucleus ovoidalis than in the adjoining optic tectum. On the other hand the large amounts of cholinesterase in the nucleus rotundus and nucleus ovoidalis indicate that they consist largely of cholinergic neurons and that they are surrounded by brain tissue containing mainly non-cholinergic neurons. Whatever these differences may mean they are so pronounced that these structures are more conspicuous in histochemical preparations than in sections stained with ordinary histological stains.

*Summary and conclusions.* Histochemical methods were used to demonstrate the presence of alkaline and acid phosphatase, phosphamidase, and cholinesterase in the brain of pigeons. It was found that there are marked differences in the enzyme contents of various structures in the brain. This observation corroborates the concept of biochemical differences between different parts or different functional and anatomical systems that has evolved from the work of a number of investigators.‡ The use of histochemical methods makes possible the more detailed study of the "Chemoarchitectonics" of the vertebrate brain which will be of interest when correlated with the cyto-, myelo- and angioarchitectonics.

[19] Feldberg, W., and Vogt, M., *J. Physiol.*, 1948, **107**, 372.

‡ The relevant literature will be discussed in another paper.

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# A Method for Measuring Removal of Bacteria from the Blood by the Various Organs of the Intact Animal.*† (17333)

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(Introduced by E. A. Stead, Jr.)

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The ability of the body to remove foreign matter from the circulation has interested investigators since the nineteenth century. Wyssokowitch[1] in 1886 noted that the cells of the vascular endothelium rapidly cleared the blood of pathogenic and non-pathogenic organisms. Aschoff[2] considered these cells as a functioning unit which he called the reticuoendothelial system. By perfusing isolated organs Manwaring[3] found that the canine liver and spleen removed organisms much more rapidly than other organs. Studies on the rapidity of removal and the acceleration of removal by immunity were carried out by Bull[4] and Wright.[5] Cannon[6] and Sullivan[7] showed that after single intravenous injections, large numbers of organisms were found in the liver and spleen with few in the brain, lungs, muscle and thyroid. Beeson[8] in his study of subacute bacterial endocarditis noted the sparsity of organisms in hepatic venous blood. The object of the present report is to describe a method of quantitative estimation of the rate of removal of bacteria from the blood stream in the intact animal.

*Methods.* Healthy 7 to 23 kg dogs and 2.6 to 3.1 kg albino male rabbits were used. Light anesthesia was maintained throughout the experiment by the use of intravenous sodium pentobarbital.

The hemolytic *Micrococcus aureus* employed was of human origin, coagulase positive, and it fermented mannite. The encapsulated Klebsiella pneumoniae type B was obtained from Dr. R. J. Dubos. The inoculum used for infusion consisted of a suitably diluted saline suspension of bacteria washed from the surface of Bacto tryptose agar after 5 to 6 hours of incubation at 37°C.

A controlled bacteremia was maintained by a continuous infusion into the femoral vein, the inferior vena cava, or the right auricle of the dog of 1 to 5 x $10^5$ hemolytic *Micrococcus aureus* organisms per minute by means of a number 6 or 8 ureteral catheter introduced into the veins. A similar infusion of approximately 1 x $10^4$ Klebsiella pneumoniae type B organisms per minute was accomplished in the rabbit using the superior vena cava. A constant rate of inflow was maintained by the use of a standard intravenous drip set or by the use of a syringe activated by a motor-driven plunger with variable gears allowing control of the rate of movement of the plunger. An initial relatively large dose of organisms was given to each animal in order to attain rapidly the desired blood bacterial count which was maintained thereafter by the constant inflow of bacteria. After 5 to 30 minutes blood samples for culture were drawn from various sites as indicated in Tables I through III.

In the dog, femoral and jugular venous and femoral arterial blood samples were drawn at approximately 15 minute intervals through an arterial-type needle left *in situ*. Hepatic

* This work was supported by the Anna H. Hanes Research Fund.

† The authors wish to express their appreciation to Dr. D. T. Smith of the Department of Bacteriology for making available the media and certain other supplies which were used.

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TABLE I.
Blood Levels of Bacteria and Percent Splanchnic Removal in Normal Dogs.

| Duration i.v. admin. bact. (min.) | 1 | | | | 2 | | | | 3 | | | | 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F.V. | F.A. | H.V. | %Eh | F.V. | F.A. | H.V. | %Eh | F.V. | F.A. | H.V. | %Eh | F.V. | F.A. | H.V. | %Eh |
| 30 | 284 | 352 | 153 | 56.5 | 515 | 457 | 98 | 78.7 | 452 | 559 | 82 | 85.3 | 421 | 335 | 42 | 87.4 |
| 45 | 356 | 377 | 27 | 92.8 | 460 | 467 | 96 | 79.3 | | 477 | 165 | 65.4 | 364 | 329 | 60 | 81.7 |
| 60 | 345 | 362 | 66 | 81.8 | 345 | 446 | 120 | 73.6 | | 444 | 147 | 66.9 | 184 | 290 | 28 | 90.3 |
| 75 | | 357 | 76 | 78.7 | 386 | 427 | 94 | 77.7 | | 484 | 79 | 83.0 | 199 | 437 | 27 | 93.8 |
| 90 | 292 | 343 | 58 | 91.5 | 229 | 224 | 43 | 80.8 | | 342 | 82 | 76.0 | 368 | 233 | | |
| 105 | 309 | 352 | 75 | 78.7 | 402 | 372 | 77 | 79.4 | | 404 | 25 | 93.8 | 249 | 468 | 17 | 96.4 |
| 120 | 238 | 247 | 60 | 75.7 | 278 | 389 | 104 | 73.3 | | | | | 405 | 480 | 28 | 94.2 |
| 135 | 214 | 251 | 73 | 70.9 | 243 | 282 | 72 | 74.5 | | | | | 353 | 446 | 20 | 95.5 |
| 150 | 244 | 292 | 61 | 79.1 | | | | | | | | | | | | |
| 165 | 104 | 284 | 60 | 78.7 | | | | | | | | | | | | |

| Duration i.v. admin. bact. (min.) | 5 | | | | 6 | | | 7 | | | 8 | | | | 18 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F.V. | F.A. | H.V. | %Eh | F.A. | H.V. | %Eh | F.A. | H.V. | %Eh | F.V. | F.A. | H.V. | %Eh | F.A. $\times 10^3$ | H.V. $\times 10^3$ | %Eh |
| 30 | | | | | 68 | 62 | 8.8 | 152 | 76 | 50.0 | 145 | 129 | 24 | 81.4 | 110 | <1 | >90.9 |
| 45 | | | | | 153 | 23 | 85.0 | 171 | 79 | 53.8 | 143 | 132 | 24 | 81.8 | | | |
| 60 | 785 | 861 | 331 | 62.0 | 79 | 20 | 74.7 | 132 | 38 | 71.2 | | | | | 81 | 17 | 79.0 |
| 75 | 826 | 903 | 426 | 53.0 | 113 | 45 | 60.2 | 129 | 56 | 56.6 | | | 80 | 18 | 44.4 | 105 | 27 | 74.3 |
| 90 | 1065 | 946 | 473 | 50.0 | | | | | | | 92 | 139 | 9 | 93.5 | 101 | 24 | 76.2 |
| 105 | | | | | | | | | | | | | | | 97 | 28 | 71.1 |
| 120 | 841 | 973 | 372 | 62.0 | | | | | | | | | | | 101 | 19 | 81.2 |
| 135 | 905 | 858 | 342 | 60.0 | | | | | | | 92 | 92 | 33 | 64.1 | | | |
| 150 | 729 | 788 | 278 | 65.0 | | | | | | | | | | | 104 | 21 | 79.8 |

F.V.—Femoral venous.
F.A.—Femoral arterial.
H.V.—Hepatic venous.
%Eh—% splanchnic removal $\frac{(\text{F.A.-H.V.} \times 100)}{\text{F.A.}}$.

TABLE II. Blood Levels of Bacteria and Percent Removal by Various Organs in Normal Dogs.

| Duration i.v. admin. bact. (min.) | Splenic vein | | | | | Pulmonary artery | | | | | Jugular vein | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | | | 10 | | 11 | | 12 | | | 13 | | | | |
| | F.A. | S.V. | %Es | F.A. | S.V. | %Es | P.A. | P.A. | F.A. | P.A. | J.V. | F.A. | H.V. | | %Eh |
| 15 | | | | | | | | | | | 139 | 123 | 14 | | 89.0 |
| 30 | | | | | | | 40 | 62 | | | 249 | 246 | 46 | | 81.3 |
| 45 | | 73 | | 208 | | | 46 | 41 | 103 | 129 | 400 | 356 | 85 | | 76.1 |
| 60 | | 63 | | 152 | 26 | 82.9 | 41 | 32 | 123 | 111 | 673 | 342 | 190 | | 44.1 |
| | | | | | | | | | | | | | | 6′ 30″ after | |
| 75 | | | | | | | 28 | 28 | | | 166,000 | 56,000 | 1500 | 97.4 | 240 × 10⁵ |
| 90 | | 32 | | 115 | 22 | 80.0 | 25 | 27 | 90 | 103 | | | | | |
| | | | | | | | | | | | | | | 18′ after | |
| 105 | | 70 | 14 | 80.0 | 126 | 6 | 95.4 | 52 | 45 | 322 | 327 | 27,000 | 25,000 | 2600 | 89.6 | 240 × 10⁵ |
| 120 | | 79 | 40 | 49.4 | | | | 46 | 38 | 426 | 411 | | | | | |
| 135 | | | | | | | | 41 | 44 | 364 | 411 | | | | | |
| 150 | | | | | | | | 28 | 16 | | | | | | | |

%Es—% splenic removal (F.A.-S.V. × 100).
F.A.

S.V.—Splenic venous.
P.A.—Pulmonary arterial.
J.V.—Jugular venous.

Other abbreviations as in preceding table.

venous, renal venous and pulmonary arterial blood samples were obtained through indwelling number 6 or 8 ureteral catheters introduced into the vessel via a jugular vein under fluoroscopic visualization. The technic of venous catheterization used in these experiments has been described by Cournand and Ranges.[9] In each instance the right lobe of the liver was catheterized. Splenic venous blood samples were obtained by repeated venipunctures or from an arterial-type needle left *in situ* under direct visualization after opening the abdomen.

In the rabbit mixed blood samples were obtained at approximately 7 minute intervals in initial preparations by repeated cardiac punctures and in subsequent preparations through an indwelling number 6 ureteral catheter introduced via a jugular vein into the inferior vena cava to the lower lumbar level. In each instance the left lobe of the liver was catheterized.

In numerous experiments the animals were sacrificed at the close of the observation period, and the position of the catheters was checked then by direct visualization.

Experiments revealed that the preliminary withdrawal of 4 ml of blood sufficed to clear the catheter of its contents so that the subsequent sample represented the blood present in the lumen of the catheterized vessel. Such clearance was done in every instance prior to the withdrawal of a blood sample for study.

Duplicate pour plates were made with blood samples of 0.5 to 1.0 ml volume in Bacto tryptose agar, serial saline dilutions of the blood being used when necessary to obtain countable plates.

*Results.* In Fig. 1 a representative experiment on a dog is charted. The bacterial counts in the femoral venous and femoral arterial blood varied somewhat during the course of each experiment. This is attributed to a slight variation in the rate of inflow of organisms and to the fact that the number of viable bacteria in the saline suspension which was administered decreased during the course of each experiment, as shown by a comparison of the

[9] Cournand, A., and Ranges, H. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 462.

TABLE III.
Blood Levels of Bacteria and Percent Splanchnic Removal in Normal Rabbits.

| Duration i.v. admin. bact. (min.) | 7 | | | 8 | | | 10 | | | 12 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | H.V. | %Eh | C | H.V. | %Eh | C | H.V. | %Eh | C | H.V. | %Eh |
| 8 | 141 | 89 | 36.9 | | | | | | | 261 | 216 | 17.2 |
| 15 | 140 | 120 | 14.3 | | | | | | | 214 | 151 | 29.4 |
| 20 | 160 | 117 | 26.9 | | | | | | | 400 | 185 | 53.8 |
| 29 | 172 | 153 | 11.0 | 386 | 214 | 44.6 | 480 | 393 | 18.2 | 283 | 214 | 24.1 |
| 36 | 190 | 156 | 17.9 | | | | 490 | 417 | 14.9 | 257 | 212 | 17.5 |
| 43 | | | | 701 | 496 | 29.2 | 647 | 505 | 21.9 | 348 | 242 | 30.5 |
| 50 | | | | 699 | 642 | 8.2 | 695 | 533 | 23.3 | | | |
| 57 | | | | 889 | 789 | 11.2 | 867 | 640 | 26.2 | | | |
| 64 | | | | | | | 904 | 803 | 11.2 | | | |

| Duration i.v. admin. bact. (min.) | 15 | | | 17 | | | 28 | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | H.V. | %Eh | C | H.V. | %Eh | V.C. | H.V. | %Eh |
| 8 | 651 | 520 | 20.1 | 360 | 338 | 6.1 | | | |
| 15 | 549 | 445 | 18.9 | 352 | 268 | 23.9 | 475 | 389 | 18.1 |
| 20 | 628 | 483 | 23.1 | | | | 564 | 494 | 12.4 |
| 29 | 615 | 486 | 21.0 | 384 | 283 | 28.0 | 733 | 545 | 25.6 |
| 36 | 545 | 481 | 11.7 | 365 | 287 | 21.4 | 726 | 632 | 12.9 |
| 43 | 606 | 549 | 9.4 | 386 | 367 | 4.9 | 787 | 665 | 15.5 |
| 50 | 758 | 613 | 19.1 | | | | | | |
| 57 | 993 | 828 | 16.6 | | | | | | |

C—Intracardiac.
H.V.—Hepatic venous.
V.C.—Vena caval.
%Eh—% splanchnic removal $\left(\frac{\text{C or V.C.—H.V.}}{\text{C or V.C.}} \times 100\right)$

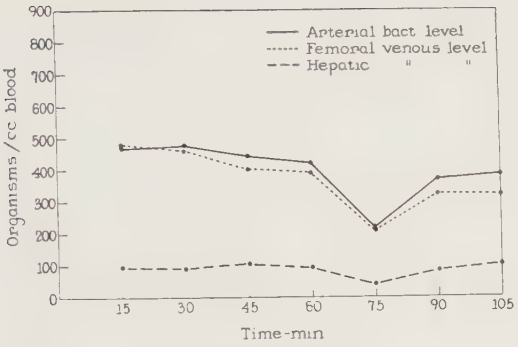


FIG. 1.

Chart showing bacteremia maintained over 105 minutes with arterial levels plotted against femoral and hepatic venous levels.

inoculum titres at the beginning and at the end of the observation period. The data permit comparison of counts on samples drawn simultaneously from different sites, however.

Table I records the counts of hemolytic *Micrococcus aureus* per ml of blood in a group of nine dogs, including one animal (No. 18) in which an arterial blood level of 1 x $10^5$ bacteria per ml was maintained throughout the experiment. The rate of removal of organism by the splanchnic circulation is recorded in percent calculated from the simultaneous hepatic venous and the femoral arterial counts. A high percentage of organisms was taken out by the splanchnic circulation, the average removal in 57 observations on nine dogs being 74 ± 16% (S.D.). An analysis of femoral venous and femoral arterial bacterial counts based on 35 observations in 6 dogs showed that the removal of organisms in the peripheral circulation of the hind extremity was variable within wide limits, averaging 12 ± 14% (S.D.).

Table II presents observations in a group of 5 dogs in which various other organs were studied for their ability to remove hemolytic *Micrococcus aureus* from the blood stream. In two dogs (Nos. 9 and 10) the bacterial counts per ml of blood from the femoral artery and the splenic vein were used to calculate the percent removal in the splenic circulation. The average removal by the spleen was 78 ± 17% (S.D.) a rate which approximates closely that of the splanchnic circulation as a whole. In 2 animals (Nos. 11 and 12) no significant difference was found on comparison of the pulmonary arterial and the femoral arterial bacterial counts. In 6 observations on a single animal (No. 13), the jugular venous counts were 24 ± 27% (S.D.) higher than the simultaneously determined femoral arterial counts. Preliminary studies in several dogs not included in the present series have indicated no consistent trend in the relationship of renal venous and superior mesenteric venous counts to femoral arterial counts.

Table III records the bacterial counts per ml of blood in a group of 7 rabbits receiving an encapsulated Klebsiella pneumonia Type B. The percent removal by the splanchnic circulation was calculated from the hepatic venous and the intracardiac or the inferior vena caval bacterial counts. Average splanchnic removal in 39 observations on 7 rabbits was 20 ± 10% (S.D.), a degree of removal which is highly significant ($p = < 0.01$).

*Discussion.* A constant intravenous bacterial infusion combined with the technic of venous catheterization provides a convenient method of determining the rate of removal of microorganisms by the organs draining directly into the caval system. The spleen must be studied by some method allowing puncture of the splenic vein. The method used here may be useful in studying the rate of removal of antigens and other particulate matter.

Cannon[6] studied the removal of the micrococcus from the blood stream by culturing weighed amounts of various organs. He showed the remarkable capacity of the animal to concentrate the injected organisms in the liver and spleen as well as the ability of these organs to destroy the micrococcus. The lungs did not concentrate the organisms after intravenous injection. Manwaring[3] found the extraction in the liver and spleen to be 80 and 60%, respectively. Beeson[8] found an average of 85% extraction in hepatic venous blood in human subacute bacterial endocarditis.

The figure for the total splanchnic removal of the hemolytic *Micrococcus aureus* in our dogs was 74 ± 16% (S.D.) and the splenic removal 78 ± 17% (S.D.). The hepatic venous and the splenic venous blood were

cleared to the same degree. The absolute efficiency of these organs cannot be determined, as the blood flow per unit of tissue is not known in either instance. The percent removal of the bacteria remained high even when the arterial levels were maintained at 50,000 and 100,000 per ml. When one considers that the splanchnic circulation receives one-fifth of the cardiac output and that approximately 75% of the organisms are removed in a single circulation, it becomes clear why such a large number of bacteria is required to maintain a blood level of 100 organisms per ml.

The tissues of the hind limb showed no consistent ability to remove organisms from the circulating blood. The percent removed in a single circulation through the hind extremity was 12 ± 14% (S.D.). No organisms were lost in circulation through the lungs of the dog.

In the present series of rabbits the total splanchnic removal of Klebsiella pneumoniae type B averaged 20 ± 10% (S.D.). The effect of variation in the parasite on the rate of removal by the host will be discussed in a later communication.

Other studies[4,5] have shown that in the course of some experimental bacterial infections, the organisms are cleared partially or completely from the blood stream after a variable period of 5 to 48 hours, the variation depending apparently on the virulence of the infecting organism and the susceptibility of the host. After a period of several hours, the blood culture may become positive again and may be associated with a progressively unfavorable clinical course leading to the death of the animal. The reason for the ineffective clearing of the blood stream in the later stage of the infection has not been determined. It may be that the removal mechanism is blocked by accumulation of organisms in the cells of the reticuloendothelial system, but this seems unlikely because the mechanism still functions efficiently at a level of 100,000 organisms per ml. The organisms may proliferate in the cells and so alter their physiologic functions. Observations on the sites from which the bacteria return to the blood stream and the state of the reticuloendothelial system at that time are needed.

*Summary.* 1. A method of constant intravenous infusion of bacteria combined with the determination of bacterial counts in the circulation at various sites by venous catheterization is described, which provides a means of determining the site and of quantitating the rate of removal of bacteria from the blood stream of the intact animal.

2. The total canine splanchnic removal of hemolytic *Micrococcus aureus* averaged 74 ± 16% (S.D.); splenic removal, 78 ± 17% (S.D.). No organisms were lost in circulation through the lungs.

3. The total rabbit splanchnic removal of encapsulated *Klebsiella pneumoniae* type B averaged 20 ± 10% (S.D.).

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## The Reversible Inactivation of Calcification *in vitro*.* (17334)

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Some aspects of normal calcification can be explained by physico-chemical concepts. For example, new calcification depends on the concentration of calcium and phosphate ions in excess of a critical product.[1-10] Furth-

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ermore the composition of the mineral matter found in bone, is related to that of the body fluids as would be expected from phase rule considerations for a solid solution.[11-14] However such physico-chemical principles do not explain why calcification takes place at specific sites and at a definite time in the life of the preosseus cells.

Calcification *in vitro* of the hypertrophic epiphyseal cartilage has served as a useful indicator of the cellular factors.[2-4,7,8,15-21] The appearance of new calcification depends on the functioning of the complete system essential for mineralization. Most of our knowledge of the "local factors" comes from such studies. Robison[15,16,18] by such *in vitro* studies indicated the importance of phosphatase. Gutman[19] has presented evidence that phosphoralative glycogenolysis is an integral part of the calcification process. However, one can demonstrate the presence of phosphatase and phosphoralative glycogenolysis in tissues other than bone. Moreover Waldman[21] has shown that calcification *in vitro* can take place when phosphatase is inactivated. Furthermore, in rickets due to strontium, calcification *in vitro* is inactivated without affecting phosphatase activity.[17,22] Thus it can not be unequivocally stated that enzymes acting on organic phosphates are responsible for mineralization.

It occurred to us that by extending the studies of the injury to calcification in strontium rickets which is reversible *in vivo* and *in vitro*,[17] the role of a system handling calcium may be explored. (In addition we were stimulated by the statement of McLean[23] "Our search for a method of producing a reasonably complete but reversible inhibition of the local mechanism has so far failed.") This observation with strontium rickets and that of Robison[24,25] who caused mineralization of the hypertrophic epiphyseal cartilage *in vitro* by replacing $Ca^{++}$ ions with $Sr^{++}$ ions suggested that strontium competes with calcium for some constituent of the bone cell necessary for calcification. The more recent experiments using radioactive strontium as an indicator of calcium metabolism are in harmony with such a concept.[26]

It was reasoned that if rachitic bone sections (produced by the usual high calcium, low phosphorus diet) were shaken with $Sr^{++}$ ions of high concentration, subsequent calcification would be inhibited by the competitive combination of strontium with a factor in the cell which is necessary for calcification. Moreover since inhibition did in fact take place, as

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TABLE I.

Reversible Inactivation of Calcification *in vitro* by $SrCl_2$ and NaCl Solutions.

| Control* No treatment | Inactivation | | | | Reactivation | | | |
|---|---|---|---|---|---|---|---|---|
| Degree of calcification† | Solution mE/L | Hr of shaking | Ca×P‡ product | Degree of calcification† | Solution mE/L | Hr of shaking | Ca×P product | Degree of calcification† |
| | $SrCl_2$-150 | 2 | 50 | 0(0), 0(0) | $CaCl_2$-100 | 1 | 50 | 2(++++), 2(++++) |
| | $SrCl_2$-150 | 2 | 50 | 0(0), 0(0) | $CaCl_2$-660 | 1 | 50 | 4(++++), 4(++++) |
| 2(++++) | $CaCl_2$-666 | 2 | 45 | 4(++++) | $CaCl_2$-666 | 1 | 45 | 4(++++), 4(++++) |
| 2(++++) | $SrCl_2$-666 | 2 | 45 | 0(0), 0(0) | $CaCl_2$-666 | 1 | 45 | 4(++++), 4(++++) |
| | $NaCl_2$-666 | 2 | 45 | 0(0), 0(0) | $CaCl_2$-666 | 1 | 45 | 4(++++), 4(++++) |
| 1½(++++) | $CaCl_2$-666 | 3 | 40 | 3(++++), 4(++++) | $CaCl_2$-666 | 1 | 40 | 4(++++), 4(++++) |
| | | | | | | | 20 | 2½(++++), 1(++++) |
| 1½(++++) | $SrCl_2$-666 | 3 | 40 | 0(0), 0(0) | $CaCl_2$-666 | 1 | 40 | 3(++++), 1½(++++) |
| | | | | | | | 20 | 1½(++++), 1½(++++) |
| 2(++++) | NaCl-666 | 3 | 40 | 0(0), 0(0) | $CaCl_2$-666 | 1 | 40 | 2½(++++), 4(++++) |
| | | | | | | | 20 | 2(++++), 0(0) |
| 0(0)‖ | | | 20 | | | | | |
| 2(++++) | $CaCl_2$-666 | 3 | 45 | 2(++++), 2(++++) | $CaCl_2$-666 | 1 | 45 | 3(++++), 4(++++) |
| | | | | | $CaCl_2$-666 | 1 | 20 | 0(0), 2(++++) |
| | | | | | $CaCl_2$-666 | 1 | 0 | 0(0), 1(+++) |
| | NaCl-167 | 3 | 45 | 1(++), 1(+) | $CaCl_2$-666 | 1 | 45 | 4(++++), 3(++++) |
| | | | | | $CaCl_2$-666 | 1 | 20 | 0(0), 1(++), 1(++) |
| | | | | | $CaCl_2$-666 | 1 | 0 | 0(0), 0(0) |
| | $SrCl_2$-167 | 3 | 45 | 0(0), 1(+++) | $CaCl_2$-666 | 1 | 45 | 4(++++), 4(++++) |
| | | | | | $CaCl_2$-666 | 1 | 20 | 0(0), 0(0) |
| | | | | | $CaCl_2$-666 | 1 | 0 | 0(0), 0(0) |
| 2(++++), 2(++++) | Saline adjusted to pH3 with HCl§ | ½ | 45 | 0(0), 0(0) | $CaCl_2$-666 | 1 | 45 | 3(++++), 1½(++++) |
| | | | | | $CaCl_2$-666 | 1 | 20 | 1(+) |
| | | | | | $CaCl_2$-666 | 1 | 0 | 0(0) |
| | Saline adjusted to pH9.6 with NaOH§ | ½ | 45 | 1(+), 1(+++) | $CaCl_2$-666 | 1 | 45 | 4(++++), 4(++++) |
| | | | | | $CaCl_2$-666 | 1 | 20 | 3(++++) |
| | | | | | $CaCl_2$-666 | 1 | 0 | 0(0) |

* Specimens incubated in calcifying solution for 18-24 hr.
Calcifying solution contains: 0.7 eq/L NaCl
0.05 eq/L NaCl
0.22 eq/L $NaHCO_3$
Ca×P product = 10 mg % Ca×5 mg % P unless otherwise noted.

† The degree of calcification is indicated as follows: 0(0) no calcification; 1(+) trace; 1(++) broken thin line; 1(+++) almost complete thin line across the provisional zone; 1(++++) complete thin line across the provisional zone; 2(++++) heavy line across the provisional zone including the primary tongues of cartilage; 3(++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4(++++) practically complete calcification of the metaphysis.

‡ Ca×P products are obtained by keeping Ca concentration constant (Ca = 10 mg %) and varying P concentration thus: Ca×P = 10×0 = 0; Ca×P = 10×2 = 20; Ca×P = 10×4.5 = 45; and Ca×P = 10×5.0 = 50.

§ Physiological saline was used; NaCl = 0.145 eq/L.

‖ Ca×P product for this control = 20.

TABLE II.
Reversible Inactivation of Calcification *in vitro* with $CuCl_2$ in the Presence of $CaCl_2$.

| Control* No treatment | Inactivation—2 hr shaking | | | Reactivation—1 hr shaking | |
|---|---|---|---|---|---|
| Degree of calcification† | Solution, mE/L $CaCl_2$ | $CuCl_2$ | Degree of calcification†‡ | Solution, mE/L $CaCl_2$ | Degree of calcification†‡ |
| 2(++++) | 150 | 0.0 | 4(++++), 4(++++) | | |
| 2(++++) | 150 | 0.1 | 4(++++), 4(++++) | 150 | 4(++++), 4(++++) |
| 2(++++) | 150 | 0.3 | 4(++++), 4(++++) | 150 | 4(++++), 4(++++) |
| 3(++++) | 150 | 0.5 | 1(++++), 1(++++) | 150 | 4(++++), 4(++++) |
| 3(++++) | 150 | 0.5 | 1(+), 0(0) | 150 | 4(++++), 4(++++) |
| 2(++++) | 150 | 0.5 | 1(+), 1(+) | 150 | 4(++++), 4(++++) |
| 2(++++) | 150 | 0.75 | 2(+++), 2(++) | 150 | 4(++++), 4(++++) |
| 2(++++) | 150 | 1.0 | 1(+), 1(+) | *660* | 4(++++), 3(++++) |
| | 150 | 1.0 | 1(+), 2(++) | 150 | 4(++++), 3(++++) |
| | 150 | 1.0 | 3(++++), 4(++++) | 150 | 4(++++), 4(++++) |
| | 150 | 1.0 | 0(0), 0(0) | 150 | 4(++++), 4(++++) |
| | 150 | 2.0 | 0(0), 0(0) | 150 | 4(++++), 4(++++) |
| | 150 | 3.0 | 0(0), 0(0) | *660* | 3(+++), 1(+) |
| | 150 | 3.0 | 0(0), 0(0) | 150 | 4(+++), 1(++++) |
| | 150 | 4.0 | 0(0), 0(0) | 150 | 1(+), 1(+) |
| | 150 | 5.0 | 0(0), 0(0) | 150 | 0(0), 0(0) |
| | 150 | 6.0 | 0(0), 0(0) | 150 | 1(+), 1(+) |
| | 150 | 10.0 | 0(0), 0(0) | 150 | 0(0), 0(0) |

* Specimens incubated in calcifying solution for 18-24 hr.
Calcifying solution contains: 0.7 eq/L NaCl
0.05 eq/L KCl
0.22 eq/L $NaHCO_3$
Ca×P product =[10 mg % Ca] [5 mg % P] = 50, unless otherwise noted

† The degree of calcification is indicated as follows: 0(0) no calcification; 1(+) trace; 1(++) broken thin line; 1(+++) almost complete thin line across the provisional zone; 1(++++) complete thin line across the provisional zone; 2(++++) heavy line across the provisional zone including the primary tongues of cartilage; 3(++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4(++++) practically complete calcification of the metaphysis.

‡ Calcifying solution same as that used previously (Table I).
Ca×P product kept constant at 10×5.0 = 50.

shown in Table I, it was further reasoned that the reverse reaction should take place, by shaking with $Ca^{++}$ ions of high strength. This actually turned out to be the case as shown in Table I. The calcification was usually more extensive than in the untreated control sections. Further experiments showed that similar reversible inactivation is possible by preliminary shaking with a high concentration of NaCl, NaCl of physiological strength adjusted to a pH of 3, and to a smaller degree with physiological saline adjusted to a pH of 9.6.

*These experiments demonstrate that it is possible to reversibly inactivate calcification in vitro* by electrolytes but do not prove that it takes place by competitive retardation. One can also explain these results as due to the simple removal of $Ca^{++}$ ions from the cell when shaking with calcium free solutions. In the reactivation step, these $Ca^{++}$ ions are replaced so that subsequent calcification can proceed. That reversible inactivation does not depend on the mere removal of $Ca^{++}$ ions was shown in the following experiments. When rachitic bone sections were shaken with 150 mE/L of $CaCl_2$ in the presence of as little as 0.5 mE/L of $CuCl_2$, calcification was inactivated as shown in Table II. When the section was subsequently shaken with the same concentration of $CaCl_2$ in the absence of the $CuCl_2$, reactivatioin took place which again was more extensive than in the untreated control sections.

These experiments indicate that $Cu^{++}$ ions combined reversibly with a necessary portion of the calcifying system. They further suggest that an important step in calcification is the combination of calcium with some constituent of the bone cell, probably part of an

enzyme system. In a calcium free medium, calcium is removed and is probably replaced by one of the competing ions. $Cu^{++}$ ions may compete so avidly for this local constituent that they preferentially combine with this factor even in the presence of large amounts of $Ca^{++}$ ions. On shaking with $CaCl_2$ solution free of the competing ions, the formation of the calcium complex is favored as an initial step to calcification.

*Summary.* It was possible to demonstrate the reversible inactivation of calcification *in vitro* of the hypertrophic epiphyseal cartilage. When rachitic bone sections are shaken with strontium chloride, sodium chloride, calcification *in vitro* is inhibited. On subsequent shaking with calcium chloride calcification *in vitro* takes place. In addition, inactivation takes place with ½ mE of cupric chloride in the presence of 150 mE of calcium chloride. On subsequent shaking with 150 mE of calcium chloride (in the absence of cupric chloride) reactivation takes place.

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## The Influence of Alloxan Diabetes on Cholesterol Atheromatosis in the Rabbit.*† (17335)

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It has long been suspected, largely on the basis of statistical studies, that humans suffering from diabetes mellitus develop arteriosclerosis not only in greater numbers than do non-diabetics, but also develop it earlier. However, conclusive experimental evidence that diabetes enhances arteriosclerosis has been lacking. It seemed to us that it might be instructive to test the influence of alloxan diabetes in the rabbit, despite the fact that the relationship of both experimental entities to their human counterparts are poorly understood.

*Methods.* The general plan of the experiment was as follows: 3 groups of rabbits, one group of which had been made diabetic with alloxan, were fed a cholesterol enriched diet; and animals from each group were examined at intervals after commencement of the diet to see if there was any difference in the time of onset or character of the vascular lesions in the different groups. Young Dutch rabbits weighing from 900 to 1400 g were given intravenous injections of alloxan monohydrate (Eastman) amounting to 80 to 100 mg per kilo of body weight daily for 2 consecutive days. Animals that were not diabetic on the third day were given similar injections on the third and fourth successive days. Although this method had been found in previous experiences with alloxan to be very effective, nevertheless in this instance a large number of animals died either during the course of the injections or within a week thereafter. Only approximately one half of the animals developed diabetes and survived long enough to be of value in the experiment.

Blood sugar determinations were performed by the method of Somogyi,[1] using a Klett-Summerson photoelectric colorimeter, both before and after alloxan injection. During the period of feeding the diet, the urines of the diabetic group were tested periodically with Benedict's qualitative reagent, and just before each animal was sacrificed, another blood

* This work was aided by a grant from The John and Mary R. Markle Foundation.

† Since preparation of this manuscript the results of G. L. Duff and G. C. McMillan (abstracted in *Am. Heart J.*, 1948, **36**, 469) have come to our attention. These results confirm and extend those presented in this paper in showing that alloxan diabetes inhibits experimental cholesterol atherosclerosis in the rabbit.

[1] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 61.

TABLE I.
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.
Group A Control Group—Basal diet and cholesterol in olive oil.

| Rabbit No. | Duration of diabetes, days | Duration of diet, days | Terminal blood sugar, mg/100 cc | Terminal blood cholesterol, mg/100 cc | Body wt Initial, g | Body wt Final, g | Atheromata Gross | Atheromata Micro. |
|---|---|---|---|---|---|---|---|---|
| 48-6 | | 38 | | | 910 | 1110 | 0 | 0 |
| 48-21 | | 40 | 132 | 576 | 1150 | 1610 | 0 | 0 |
| 48-25 | | 41 | 110 | 864 | 823 | 1160 | 0 | + |
| 48-22 | | 48 | 120 | 864 | 1000 | 1640 | ++ | ++ |
| 48-20 | | 49 | | | 1080 | 1790 | + | + |
| 48-4 | | 58 | | 1120 | 1060 | 1535 | 0 | + |
| 48-8 | | 72 | | 1025 | 1500 | 2225 | +++ | +++ |
| 48-10 | | 72 | | 1425 | 1425 | 1755 | ++ | ++ |
| 48-15 | | 72 | | 837 | 1961 | 2200 | +++ | +++ |
| Avg | | 54 | 121 | 959 | 1212 | 1670 | | |
| Avg of control values | | | 120 | 141 | | 1212 | | |
| % change | | | +1 | +580 | | +38 | | |

sugar determination was performed. Blood cholesterol values were determined on each animal of this group before alloxan injection and before sacrificing according to the method of Bloor, Pelkan and Allen.[2] Those rabbits which manifested a blood sugar of 200 mg% or higher were accepted as diabetic. A total of 8 rabbits satisfying this criterion and showing persistent glycosuria for the duration of the experiment were thus obtained.

Nine rabbits of similar strain, age, and original body weight were used as a control group. Blood sugar values were determined on 3 of these animals before beginning the diet and before sacrificing, and blood cholesterol levels were determined on all except 2 animals at the same times.

A third group consisted of 3 rabbits which had received 2 injections of alloxan and had manifested a temporary glycosuria and hyperglycemia, but the urines of which later became sugar free.

For the cholesterol enriched diet, Pfansteil cholesterol suspended in olive oil to a concentration of 6% was added to Rockland Rabbit Ration in such amounts that each rabbit received between 0.3 and 0.6 g per day.

All rabbits of the 3 groups were then fed this cholesterol enriched diet and animals from each group were sacrificed at various intervals after commencement of the diet. Complete autopsies were performed, with especial attention being paid to the heart, aorta, and great vessels. The presence of atheromata in these organs is summarized in the tables. It may be mentioned here that the pathological changes with respect to the other organs were similar to those previously described for alloxan diabetes and cholesterol atheromatosis separately.

*Experimental observations.* Table I summarizes the data on the control group. It is seen that there was a consistent increase in blood cholesterol values, the increase roughly paralleling the duration of the diet, and the average increase being approximately seven fold. The weight increase was also consistent. The item of chief interest is the fact that in all animals fed the diet 41 days or longer, there was gross or microscopic evidence of atheromata, the severity of the lesions roughly corresponding to the duration of the diet. The atheromata were in all respects similar to those previously described in this condition.

In Table II are seen the summarized data on the diabetic animals. Among these the elevation in blood cholesterol was very irregular, but the average is higher than the average for Group A, and in 3 cases it is considerably higher than that of any single rabbit in Group A. All of these rabbits except two (48-26 and 48-27) lost weight. Much to our astonishment, when these animals were autopsied, only one showed any evidence of

2 Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191.

TABLE II.
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.
Group B Diabetic Group—Same as control plus alloxan.

| Rabbit No. | Duration of diabetes, days | Duration of diet, days | Terminal blood sugar, mg/100 cc | Terminal blood cholesterol, mg/100 cc | Body wt Initial, g | Body wt Final, g | Atheromata Gross | Atheromata Micro. |
|---|---|---|---|---|---|---|---|---|
| 47-7 | 22 | 18 | 365 | 503 | 940 | 763 | 0 | 0 |
| 47-12 | 20 | 20 | 297 | | 1060 | 550 | 0 | 0 |
| 47-11 | 31 | 31 | 279 | 2000 | 1213 | 1175 | 0 | 0 |
| 47-8 | 41 | 41 | 268 | 665 | 1360 | 550 | 0 | 0 |
| 48-26 | 43 | 41 | 205 | 960 | 1000 | 1300 | 0 | 0 |
| 48-27 | 48 | 48 | 190 | 349 | 692 | 1380 | 0 | 0 |
| 48-11 | 68 | 65 | 300 | 2000 | 1220 | 650 | 0 | 0 |
| 47-10 | 98 | 98 | 319 | 3820 | 1010 | 910 | + | + |
| Avg | 47 | 46 | 278 | 1287 | 1062 | 910 | | |
| Avg of control values | | | 117 | 132 | | 1062 | | |
| % change | | | +138 | +875 | | —14 | | |

TABLE III.
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.
Group C—Alloxan injected—non-diabetic.

| Rabbit No. | Duration of diabetes, days | Duration of diet, days | Highest blood sugar, mg/100 cc | Terminal blood cholesterol, mg/100 cc | Body wt Initial, g | Body wt Final, g | Atheromata Gross | Atheromata Micro. |
|---|---|---|---|---|---|---|---|---|
| 48-24 | ? | 40 | 180 | 368 | 1070 | 1530 | + | + |
| 48-28 | ? | 52 | 230 | 368 | 910 | 1570 | +++ | ++ |
| 48-3 | ? | 69 | 157 | 672 | 985 | 1650 | ++ | ++ |
| Avg | | 54 | 189 | 469 | 988 | 1583 | | |
| Avg of control values | | | 120 | 140 | | 988 | | |
| % change | | | +58 | +235 | | +60 | | |

TABLE IV.
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.
Summary of data on all rabbits fed diet for 40 days or longer.

| Group | No. with atheromata: No. in group | Percent positive |
|---|---|---|
| A. Control | 7/8 | 88 |
| B. Diabetic | 1/5 | 20 |
| C. Alloxan injected, non-diabetic | 3/3 | 100 |

atheromata—and this rabbit (47-10) had been fed the diet for 98 days.

In Table III are the data on the alloxan-injected, non-diabetic animals, in which it is seen that they behaved as did the controls in Group A.

Table IV summarizes the incidence of atheromata in all rabbits fed the high cholesterol diet for 40 days or longer. These figures become even more impressive when one recalls that the one diabetic animal had developed only minimal lesions after 98 days on the diet.

*Discussion.* In criticism it may be pointed out that the number of animals is very small and that there were no diabetic animals kept as controls. These defects were due to the difficulties encountered in inducing alloxan diabetes. However, with respect to the latter point, in previous experiences by one of the authors with alloxan diabetic rabbits several animals kept for as long as 11 months showed

no atheromata and no reports of finding atheromata in alloxan diabetic rabbits have appeared in the literature.

In discussion we would like to mention one observation concerning the food intake of these animals. It was noted soon after the beginning of the experiment that, contrary to previous experiences with regular diets in which diabetic animals consumed much more food than normal rabbits, these diabetic rabbits did not eat the oily diet nearly so enthusiastically as did the controls. A greater amount of cholesterol had to be added per unit weight of ration to insure that each rabbit received at least 0.3 g daily. Facilities did not permit us to determine the exact daily consumption of each rabbit. The control animals, therefore, may have received somewhat more cholesterol per day than did the diabetic animals, but it is certain that they did not receive more than twice as much. It is our opinion that the difference in intake was not sufficiently great to account for the very great difference in time of onset of atheromata.

Whatever the mechanism, and despite the above mentioned criticisms, we feel that these findings clearly indicate that under the conditions of this experiment alloxan diabetes retards in time and degree the development of cholesterol atheromatosis in the rabbit, and that it represents a conclusive demonstration that hypercholesterolemia of itself does not lead to the formation of atheromata.

*Summary.* Alloxan diabetic rabbits, when fed a cholesterol enriched diet, developed blood cholesterol levels much higher than did normal rabbits fed the same diet. Atheromata appeared consistently in controls (and also in alloxan injected, nondiabetic rabbits) after 41 days on this diet, but only to a slight degree in one diabetic rabbit fed the same diet for 98 days. There was no qualitative difference in the character of the lesions in the 3 groups.

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## Studies on the Pathogenesis of Experimental Necrotizing Arteritis.* (17336)

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Previous studies from this laboratory[1-3] have shown that acute necrotizing arteritis can be produced with regularity in dogs by feeding a specified high fat diet for a period of 8 weeks or longer then sacrificing them through renal damage. Three factors, time, diet and renal damage, seem to be essential in the production of these lesions.

Methods have been published in detail previously.[2] Briefly these consist of feeding healthy adult dogs a high fat diet for a period of 8 weeks or longer, then sacrificing them through renal damage. The active "dietary factor" is found in cod liver oil but is apparently not unique to cod liver oil. The oil may be added to a kennel diet of unselected table scraps or to a "standard diet", consisting of calves liver (raw wet weight), 32 parts; cane sugar, 25 parts; corn starch, 25 parts; butter, 12 parts and cod liver oil, 6 parts, with equal results. Renal damage has been produced by any one of 3 ways: 1) uranium nitrate subcutaneously; 2) mercuric chloride intravenously; and 3) bilateral nephrectomy; all equally effective.

In all experiments to date, renal damage has been essential. To determine whether or not damage to tissues and organs other than the kidney would precipitate the arterial le-

* This work was aided by a grant from The John and Mary R. Markle Foundation.

1 Holman, R. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 307.

2 Holman, R. L., and Swanton, M. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 87.

3 Holman, R. L., *So. Med. J.*, 1949, **42**, 108.

TABLE I.
Studies on the Pathogenesis of Experimental Necrotizing Arteritis.

| Group | Dog No. | Period on standard diet before injury, wks | Type of injury | Total dose | Survival interval, days | Terminal NPN | Arterial lesions |
|---|---|---|---|---|---|---|---|
| Group A | 47-41 | 10 | Chloroform anesthesia | 28 hr | 20 | 50 | 0 |
| Chloroform | 47-46 | 9 | " | 10 hr | 6 | 68 | 0 |
| Injury | 47-50 | 9 | " | 13 hr | 9 | 50 | 0 |
| to | 47-74 | 13 | " | 12 hr | 7 | 5* | 0 |
| liver | | | | | | | |
| Group B | 47-73 | 12 | Turpentine | 32 cc | 18 | 37* | 0 |
| Suppuration | 47-64 | 13 | Peritonitis | | 21 | 12* | 0 |
| | 48-94 | 14 | Cellulitis | | 3 | 58* | 0 |
| Group C | 49-34 | 9 | Bilateral ureteral ligature | | 6 | 255*† | 0 |
| Bilateral ureteral ligature | 49-35 | 9 | " | | 6 | 168*‡ | 0 |

* Blood urea nitrogen instead of non-protein nitrogen.
† Terminal blood serum phosphorus 21 mg per 100 ml.
‡ Terminal blood serum phosphorus 13 mg per 100 ml and terminal blood carbon dioxide combining power 38 volumes %.

sions in properly fed dogs, the following two series of experiments were undertaken:

1) *Chloroform injury to the liver.* Four properly fed dogs (Table I, Group A) were subjected to light chloroform anesthesia for periods totalling 10-28 hours in 6-20 days. Marked liver injury was indicated by the appearance of jaundice and was confirmed by histological study of the liver. None of these 4 dogs showed gross or microscopic lesions in the arterial system.

2) Suppuration (Table I, Group B). One dog (47-13) was given repeated subcutaneous injections of turpentine for a total dose of 32 cc in 18 days. Two of the sterile abscesses broke down and each discharged about 100 cc of sanguinopurulent material. The total white blood cell count rose progressively to 45,500. At the time of autopsy there were 12 subcutaneous abscesses estimated to contain a total of 400 cc of similar purulent material but no arterial lesions.

The experimental induction of intestinal obstruction was attempted in two other dogs but neither operation accomplished its intended purpose. Instead, in one dog (47-64) the ligature (umbilical tape about 1 foot above the ileocecal valve) cut through the bowel and the dog died with generalized peritonitis on the 21st day. In the second attempt (Dog 48-94) the small intestine about 2 feet below the ligament of Treitz, was divided between ligatures and the closed proximal end was sutured into the subcutaneum of the abdominal wall. This proximal stump "blew out" and the dog died on the 3rd day with massive fecal contamination of the subcutaneum (associated with cellulitis and fat necrosis) that extended from the hypogastrium to below the inguinal ligament. Despite the massive necrosis and suppuration no arterial lesions were found in either of these two dogs.

Since severe renal damage has been a prerequisite for the arterial lesions the following experiments were undertaken in an attempt to define more accurately what phase or part of "renal insufficiency" was responsible for the subsequent anatomical changes in the arterial system.

*Bilateral ureteral ligations.* Under nembutal anaesthesia both ureters of 2 properly fed dogs (Table I, Group C) were doubly ligated and divided between ligatures at a level midway between the uretero-pelvic and ureto-vesical junctions. Each dog stopped eating on the 2nd post-operative day and each showed a progressive azotemia, acidosis, phosphatemia, and "uremia". At autopsy on the 6th post-opera-

tive day, each had about 300 cc of clear fluid in the peritoneal cavity; but neither dog had arterial lesions. Histological study of the kidneys showed early hemorrhage and necrosis about the angles of the calyces and early hydronephrosis. The collecting tubules were moderately dilated but the remaining tubules were remarkably well preserved.

*Discussion.* These experimental observations reaffirm the importance of the kidney in the pathogenesis of arterial lesions. All of our results to date indicate that some derangement of renal function is prerequisite to the development of arterial lesions. Other workers[4-6] have produced arterial lesions with various types of renal insufficiency, but none of these workers has found it necessary to control the diet of his experimental animals. Whereas, in our experience, "standard renal insufficiency" alone resulted in "typical arterial lesions" in only 5 of 130 dogs (4%), the combination of "standard high fat diet" and "standard renal insufficiency" resulted in "typical arterial lesions" in 36 to 40 dogs (90%). This discrepancy between our results incriminating a "dietary factor" and the results of others ignoring a "dietary factor" may, in part, be related to the definition of "typical arterial lesions", for we do not consider some of the hemorrhagic lesions illustrated in previous publications[4,5] "typical" of our experimental lesions which are predominately necrotizing in character and only occasionally associated with gross hemorrhage.

If, as the above results indicate, "standard high fat diet" and "standard renal insufficiency" are *both* necessary for the production of "typical arterial lesions," the problem simmers down to what role the kidney plays in the metabolism of the potentially noxious fatty substance or substances contained in the specified high fat diet. During the 2 months or more of high fat feeding, the kidneys—and especially the epithelial cells lining the loops of Henle along with those of the distal portion of the proximal convoluted tubules—become distended with sudanophilic material. This condition is still compatible with normal life, for the high fat diet can be fed indefinitely and no predictable changes in the vascular system are ever observed unless the kidneys are damaged. Anytime after 2 months of such a diet "standard renal insufficiency" is regularly followed by "typical arterial lesions". It can be surmised that the intact kidney elaborated something necessary for the metabolism of the noxious lipids and that when the kidney is severely damaged the noxious lipids (or metabolic by-products thereof) pile up to "explosive" levels as manifested by the arterial lesions.

The factor common to all the methods used for the production of "standard renal insufficiency" that have resulted in "typical arterial lesions" is massive "damage" to the proximal convoluted tubules in a relatively short period of time (massive coagulative necrosis with uranium nitrate and mercuric chloride and mass exclusion in the case of bilateral nephrectomy). The experiments reported in this paper show that severe damage to tissues and organs other than the kidneys does not precipitate arterial lesions in properly fed dogs and that bilateral ureteral ligation—despite degrees of azotemia, phosphatemia, acidosis, and "uremia" corresponding to those produced by "standard renal insufficiency"—is likewise ineffective in precipitating arterial lesions in properly fed dogs.

The simplest explanations that we have been able to formulate for these unanticipated findings are: (1) the proximal convoluted tubules elaborate something (lipase?) necessary for the proper utilization of certain lipid substances or (2) the integrity of the proximal convoluted tubules is necessary for the neutralization of certain toxic substances (amines?). These hypotheses are based in large part upon the fact that six days after bilateral ureteral ligation the epithelium lining the proximal convoluted tubules is fairly well preserved whereas six days after "standard renal insufficiency" this epithelium appears to

4 Wintenitz, M. C., Myon, E., Walters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **12**, 623.

5 Wintenitz, M. C., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **13**, 15.

6 Goldblatt, H., *Harvey Lectures*, 1937-38, **33**, 237.

be almost completely destroyed. If these experimental findings are confirmed and if our reasoning is valid, the identification of these hypothetical substances (lipases ?, amines ?) might help clarify the time-honored relation of the kidney to arterial lesions.

*Summary.* Previous studies have shown that the combination of "standard high fat diet" plus "standard renal damage" resulted in typical arterial lesions in 36 of 40 dogs (90%). The data presented in this paper indicate: (1) "Standard high fat diet" plus damage to organs and tissues other than the kidney (chloroform injury to the liver, turpentine abscesses of subcutaneum, and bacterial infections) failed to produce arterial lesions and (2) "Standard high fat diet" plus renal insufficiency produced by bilateral ureteral ligation also failed to produce arterial lesions. These studies re-emphasize the importance of the kidney in the pathogenesis of necrotizing arteritis and indicate that azotemia *per se* is not the "renal factor". They suggest the possibility that the epithelium of the proximal convoluted tubules elaborates one or more substances necessary for the proper utilization of certain lipid substances.

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## Diurnal Variations in the Mating Behavior of Male Rats.* (17337)

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Female albino rats usually come into estrus and display their most active mating behavior during the night;[1] and animals maintained under artificial illumination with a reversed light-dark cycle become receptive during the solar day.[2] The present investigation tested the hypothesis that a comparable rhythm of sexual responsiveness occurs in males of the same species.

*Methods.* Twenty-seven adult, virgin males of the Sprague-Dawley strain were divided into 2 groups that were kept in different halves of a windowless room which was bisected by a black curtain. From 11 P.M. to 11 A.M. one half of the room was dark and the other was illuminated by 2 100 watt bulbs. From 11 A.M. to 11 P.M. the lighting conditions in the two halves of the room were reversed. For approximately 3 weeks before testing began and throughout the remainder of the experiment all animals lived under identical conditions save for the fact that for Group I it was "day" from 11 P.M. to 11 A.M. and for Group II this same period was "night."

The first 2 mating tests were conducted between the hours of 3 P.M. and 7 P.M. The tests occurred, therefore, in the middle of the "night" as far as Group I was concerned, and in the middle of the "day" for Group II. The third and fourth tests were made from 3 A.M. to 7 A.M., thus reversing the temporal placement employed in tests 1 and 2. Mating tests for each male were spaced 3 days apart to allow for complete recovery from effects of sexual exercise. The stimulus animals were spayed, Sprague-Dawley females brought into heat by injection of estrogen and progesterone.† Methods of testing sexual behavior were the same as those employed by the senior author in previous studies.[3] A test lasted for 10 minutes if there was no coital behavior, and for 10 minutes from the time of the first complete or incomplete copulation if either response occurred.

*Results.* The experimental results are sum-

* This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

† The hormone preparations were generously supplied by Dr. Edward Henderson of Schering Corporation, Bloomfield, N. J.

1 Ball, J., *Comp. Psychol. Monogr.*, 1937, **14**, 1.

2 Beach, F. A., *J. Comp. Psychol.*, 1938, **26**, 355.

3 Beach, F. A., *J. Exp. Zool.*, 1944, **97**, 249.

TABLE I.
Sexual Behavior of Two Groups of Male Rats Tested in Opposite Phases of Diurnal Light Cycle.

| | Group I (N = 14) | | Group II (N = 13) | |
|---|---|---|---|---|
| Measure of sexual performance | Tested in dark phase | Tested in light phase | Tested in light phase | Tested in dark phase |
| % of group copulating | 85.7 | 71.4 | 23.1 | 92.3 |
| % of group ejaculating | 78.6 | 64.3 | 7.7 | 92.3 |
| Avg frequency of copulation* | 9.3 | 8.4 | 8.3 | 10.6 |
| Avg frequency of ejaculation* | 1.2 | 1.0 | 1.0 | 1.3 |
| Avg seconds delay before first copulation | 46.0 | 66.0 | 66.0 | 40.0 |

* Average frequencies based on performance of animals showing the response at least once in the test.

marized in Table I, which is based upon the average performance of each group during tests 2 and 4. Comparable group differences were apparent in the first and third tests, but it seems preferable to restrict our attention to behavior occurring in the second test of each series because this allows for preliminary adjustment to handling and testing during the particular phase of the light cycle that happens to be involved. The 14 animals in Group I were observed during the dark phase of the cycle in tests 1 and 2, whereas for the 13 males in Group II the first 2 tests occurred in the middle of the lighted portion of the 24-hour period. Group I was by far the most active sexually. Rats tested during the dark phase of the cycle were more likely to copulate and to ejaculate than animals placed with females in the light phase. And when coital behavior appeared it involved a higher frequency of intromissions and ejaculations in the case of the males tested during their "night." Considering only those animals that showed mating responses, the average latency, or delay preceding initiation of sexual contact was shorter for Group I than for Group II.

The time of testing was shifted by 12 hours during the third and fourth tests, and as a result the performance of animals in Group II improved appreciably. The proportion of the group showing copulatory responses increased by 69.2%, and the number reaching the point of ejaculation rose from 7.7 to 92.3%. The mean frequency of completed copulations and ejaculations increased, and the average duration of latencies was reduced. There is little doubt that most if not all of the increased sexual activity was due to changing the time of testing from the light to the dark phase of the diurnal light cycle.

Theoretically it might have been anticipated that the performance of Group I would deteriorate during the third and fourth tests, since at this time these animals were being tested during their "day." Some decrease in activity did occur and all changes were in the expected direction, but the scores remained well above those recorded for Group II during the first 2 tests. When Group I rats were shifted from "night" to "day" tests, the proportion of animals copulating at least once decreased, as did the percentage of the group achieving ejaculation. The average frequencies of copulations and ejaculations were similarly reduced, and the mean delay before initiation of sexual responses grew appreciably longer.

*Discussion.* Comparisons of the performance of the 2 experimental groups in test 2 fully confirms the hypothesis that male rats are sexually more active during the dark phase of their light-dark cycle. Care was taken to hold testing conditions constant for all males, and the females used as stimulus objects for the two groups were equally active and receptive. The pronounced change in the behavior of Group II animals when their tests were shifted from the light to the dark phase of

the artificial light cycle is added proof of the greater sexual potency during the "nocturnal" hours.

Female rats tend to come into heat during the night because of the associated rhythm of secretion of gonadal hormones. This rhythm in turn reflects the cyclic secretion of gonadotrophic hormones by the anterior pituitary, the functions of which are influenced by periodic increase and decrease in the amount of environmental illumination as well as concomitant changes in external temperature. Regular fluctuations in hypophyseal activity also affect the secretory behavior of the thyroid and probably of still other endocrine glands. As a result, the albino rat displays a diurnal rhythm of energy metabolism which begins to increase at approximately noon, reaches a maximum around 9 P.M. and progressively declines to the next noon. If rats are placed under constant illumination and fed at frequent intervals, the metabolic rhythm is abolished.[4]

There is no reason to believe that the rat testis secretes androgen at significantly different rates during different times of day, and even if it did so the behavioral response to alteration in the concentration of testicular hormone is too slow to account for the diurnal variations that characterize the male's mating performance. The heightened sexual excitability shown by male rats during the dark phase of the illumination cycle probably is due to the nocturnal increase in basal metabolism and to a consequent increase in responsiveness to all kinds of exteroceptive stimulation.

The sexual activity of males in Group I decreased only slightly when the time of testing was changed from the dark to the light phase of the 24-hour period. In all probability the continued high level of responsiveness was due to the effects of the first two tests when these animals were mated during their "night." In tests 1 and 2 the majority of Group I males gained a considerable amount of sexual experience. They became accustomed to receiving receptive females in the observation cage and achieved orgasm as a result of the subsequent copulatory contact. The resultant conditioning "carried over" to tests 3 and 4 and tended to compensate in part for the lower reactivity that usually obtains during the "day" segment of the diurnal cycle. Unfortunately it was necessary to begin the second series of tests within a week after the first series closed. An appreciably longer interval would probably have decreased the positive carry-over in Group I and produced appreciably lower sex scores for these animals when they were tested in the lighted phase of the illumination cycle.

*Conclusions.* Twenty-seven sexually-inexperienced male rats were maintained under artificial lighting conditions. The living cages were illuminated for 12 hours and darkened for a similar period. All animals were observed in mating tests with receptive females. Males tested during the dark phase of their diurnal cycle were much more active sexually than others tested in the lighted half of the period. When tests were shifted from the light to the dark phase, mating reactions increased promptly. In response to the reverse change sexual activity was reduced. Lowering of responsiveness which accompanied a shift from dark to light phase of the cycle was compensated in part by the effects of experience gained during previous tests conducted in the dark phase. It is concluded that the male rat's tendency to engage in sexual behavior is greater at night than in the day, and that the difference is probably due to the higher metabolic rate which characterizes the early nocturnal portion of the diurnal cycle.

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# Circulating Antibodies in Vitamin Deficiency States. Pantothenic Acid Deficiency.* (17338)

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In a previous paper, we have reported that pantothenic acid-deficient rats show a severe impairment of antibody response.[1] This finding was in contrast to the observation of Stoerk, Eisen, and John that pantothenic acid deficiency did not affect the level of circulating antibodies.[2] We have attempted to explain this difference in results on the basis that the sheep erythrocytes employed by Stoerk *et al.* were a poorer antigenic stimulus for hemagglutinin production in the rat than the human red blood cells employed by us. Differences in the immunization procedures were also noted. Stoerk, on the other hand, believes that this difference may be attributed to the fact that we were dealing with a relatively more severe pantothenic acid deficiency.[3] Our failure to utilize inanition controls for the pantothenic acid deficient rats, as well as the supposed inadequacy of "paired-fed" controls as contrasted with "paired-weighed" controls were also stressed by this author.[3,4] Some of these points have already been discussed.[5]

The present experiments were planned to determine the effects of varying degrees of pantothenic acid deficiency upon antibody production in the rat utilizing "paired-weighed" animals as inanition controls.

*Experimental.* Fifty-eight male weanling albino rats of the Sprague-Dawley strain were distributed into 3 groups as indicated in Table I. The animals were housed individually in wide-meshed, screen-bottom cages and weighed daily. Daily food consumption records were taken. All rats were fed a basal diet of the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,[6] 4.00; cod-liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; 1-inositol, 0.03; p-aminobenzoic acid, 0.01; and 2-methyl-1, 4-naphthoquinone, 0.001. All rats received additional vitamins in the form of a daily pill. Each of the pills given to the control animals supplied the following vitamins: thiamin, 40 $\gamma$; riboflavin, 60 $\gamma$; pyridoxine, 50 $\gamma$; nicotinic acid, 100 $\gamma$; biotin, 1 $\gamma$; folic acid, 1 $\gamma$; and calcium pantothenate, 300 $\gamma$. For the pantothenic acid deficient rats, calcium pantothenate was omitted from the pill. The deficient rats were fed the basal diet *ad libitum*. However, each control rat was fed a restricted amount of the basal diet sufficient to maintain its weight equal to that of its pantothenic acid-deficient partner (paired-weighing). After 3, 5, and 7 weeks on experiment, the rats of each group were immunized. A 10% suspension of washed Group O, Rh positive human erythrocytes in physiological saline was injected intraperitoneally as antigen. An initial dosage of 0.5 ml of the red blood cell suspension was followed by 2 one ml injections on alternate days. Five days after the final injection the rats were bled from the heart under ether anesthesia and the sera tested for agglutinin titers as described previously.[1] Immediately after bleeding, the thymus of each rat was removed and weighed.

*Results.* The individual hemagglutinin titers are recorded in Table II. It is evident that

* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1 Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 137.

2 Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

3 Stoerk, H. C., *Nutrition Rev.*, 1948, **6**, 191.

4 Stoerk, H. C., private communication.

5 Axelrod, A. E., Carter, B. B., and McCoy, R. H., *Nutrition Rev.*, 1948, **6**, 351.

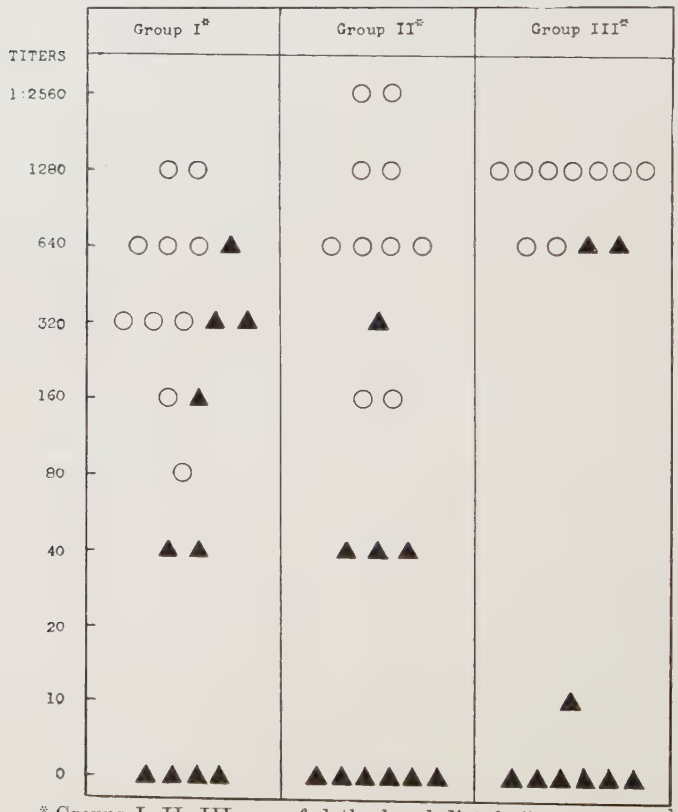
6 Jones, J. H., and Foster, C., *J. Nutrition*, 1942, **24**, 245.

TABLE I.
Summary of Growth, Food Consumption, and Thymus Weight Data.*

| Type | No. of rats | Body wt† | | Thymus‡ wt | Daily food consumption† |
|---|---|---|---|---|---|
| | | Initial | Final§ | | |
| | | Group I* | | | |
| Control | 10 | 42 | 83 | 195 | 4.4 |
| Pantothenic acid-deficient | 10 | 42 | 84 | 116 | 4.8 |
| | | Group II* | | | |
| Control | 10 | 43 | 83 | 140 | 4.0 |
| Pantothenic acid-deficient | 10 | 43 | 84 | 78 | 4.4 |
| | | Group III* | | | |
| Control | 9 | 42 | 94 | 90 | 3.9 |
| Pantothenic acid-deficient | 9 | 43 | 96 | 69 | 4.7 |

* Groups I, II, III were fed basal diet 3, 5, and 7 weeks respectively prior to immunization
† Group average in g.
‡ Group average in mg.
§ At the time of bleeding.

Table II.
Individual Hemagglutinin Titers.




* Groups I, II, III were fed the basal diet 3, 5, and 7 weeks, respectively, prior to immunization.
○—Control animals.
▲—Pantothenic acid-deficient animals.

the antibody response was decreased in the pantothenic acid-deficient rats of all groups. In no instance, did the titer of a deficient rat equal that of its "paired-weighed" control. The deleterious effect of a mild pantothenic acid deficiency is evidenced by the decreased titers of the animals in Group I which were on experiment for only 3 weeks prior to immunization. This observation lends no support to the explanation offered by Stoerk for the discrepancy in the findings of the two laboratories. The comparatively high antibody content of the "paired-weighed" controls is further evidence against the role of inanition in the decreased antibody response of pantothenic acid-deficient rats.

In the course of further studies on the mechanism of action of pantothenic acid we have determined the antibody response in 44 pantothenic acid-deficient rats and 29 suitable controls. The results obtained are in excellent agreement with those recorded in this as well as in a previous paper.[1] All of the evidence to date firmly implicates pantothenic acid as a vital factor in the antibody response to the antigenic stimulus of human erythrocytes.

The thymus weights of the pantothenic acid-deficient rats were lower than those of the control group (Table I). These findings are in agreement with those made by Stoerk *et al.*[2]

*Summary.* (1) Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in rats fed a pantothenic acid-deficient diet for 3, 5, and 7 weeks. "Paired-weighed" animals served as inanition controls.

(2) Marked impairment of antibody response was observed in all pantothenic acid-deficient groups.

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## Selective XYZ Factor in C57 Black Mammary Carcinoma Eo771. (17339)

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The XYZ phenomenon may be defined as the increased incidence and more rapid increment of local and metastatic transplanted tumors following the prior injection in the same or distant sites of an extract from the homologous tumor.[1-4] The factor in the Brown-Pearce rabbit tumor responsible for the phenomenon is selective, filtrable (Berkefeld "V") and thermolabile (56°C);[5-7] that in Bashford mouse mammary carcinoma 63[8,9] is likewise highly selective or specific.[10-12]

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1 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 816.

2 Casey, Albert E., *Am. J. Cancer*, 1934, **21**, 760.

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5 Casey, Albert E., *Am. J. Cancer*, 1936, **26**, 276.

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8 Haaland, M., *Proc. Roy. Soc. London*, 1910, **82**, 293; *Lancet*, 1910, **1**, 787.

9 Leitch, A., *Lancet*, 1910, **1**, 991.

10 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 674.

11 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1025.

12 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 663.

Brown-Pearce and Bashford tumor tissues aseptically removed from the host, frozen immediately and kept frozen at 0-24°F until the cells are no longer viable uniformly contain the respective XYZ factors in great quantity; cultures for bacteria have been sterile[8,9,13] and the Brown-Pearce tumor tissue has been free from Virus III, rabbit pox and other known viruses.[14] No reaction appears at the site of single or repeated injections of the XYZ materials. Similarly prepared extracts of normal tissues or of other tumors even in the same species when injected prior to transplantation of Bashford 63 or the Brown-Pearce tumor have not elicited the XYZ phenomenon.[5,13,15]

Recently Snell, Cloudman, Failor, and Douglass,[16] and Snell, Cloudman, and Woodworth[17] have reported similarly planned experiments using inbred strains of mice. Their data indicate to us enhancement of homologous tumor growth entirely acceptable to the above definition of the XYZ phenomenon by factors present in C57 Black mouse mammary carcinoma Eo771 and Line A mouse mammary carcinoma 15091a. Their technic consisted in freezing and storing the tumor tissue with lyophilization as an added routine procedure. Lyophilization has been found to be an effective method of storing the XYZ factor from the Brown-Pearce tumor.[18] Desiccation after freezing of the Brown-Pearce tumor by the methods current in 1930, resulted in some loss in the potency of the XYZ factor,[1] and the more modern lyophilization methods such as those used by Snell, Cloudman, and Woodworth are a great improvement in the study of the XYZ factors.

The present communication reports experiments by us with Eo771 and correlates our results with those previously obtained by Snell, Cloudman, and Woodworth.

*Material and methods.* The animals were 250 C57 black mice received from Carworth Farms, New City, New York over a period of one year in batches of 40 (animals of each batch of same sex). On receipt the animals were placed in metal self-feeder mouse boxes 10 to a box, and alternate boxes marked "control" and "experimental" respectively. The animals were allowed from 1-4 months to mature and become adjusted to the new environment. The animal quarters were air conditioned throughout the year, temperature set at 70°F and humidity at "ideal." Some 8 experimental and 9 control mice died of intercurrent disease over an average 6-8 months observation period (loss 6.8%) and usually before experiments were initiated. Since the diagnosis in each was checked by necropsy and histologic examination these 15 mice were eliminated from the series. In no instance did injection of XYZ material lead to local infection or abscess. Except in 2 experiments (40 mice) no evidence of infection appeared at the sites of tumor transplantation. In the 2 experiments above mentioned abscesses (4-5 mm in diameter) appeared at the site of tumor transplantation in about 30-40% of control as well as experimental animals. In each instance transfer had been made from a large growth having considerable necrosis. The number of takes in the 2 experiments was less than normal, although good tumors eventually grew from most of the animals. No animal died because of the intradermal abscesses, and all lesions were healed for the most part by 15 days.

There were 12 separate experiments with equal numbers in control and experimental series with one exception in which there were 20 controls instead of the usual 10 (Table I). Measurements of the tumors in 3 dimensions in the controls and experimental animals were made concomitantly at intervals after inoculation of the tumor tissue. Although the trends remained uniform from the first meas-

[13] Drysdale, George R., and Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 306.

[14] Pearce, Louise, and Casey, Albert E., 1933 (unpublished experiments, The Rockefeller Institute for Medical Research).

[15] Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 731.

[16] Snell, George D., Cloudman, Arthur M., Failor, E., and Douglass, P., *J. Nat. Cancer Inst.*, 1946, **6**, 303.

[17] Snell, George D., Cloudman, Arthur M., Woodworth, Eliz., *Cancer Research*, 1948, **8**, 429.

[18] Drysdale, George R., Ross, Gordon L., Skipper, Howard, Edwards, P. C., and Casey, Albert E., unpublished experiments.

TABLE I.
Experiments with XYZ Factor from Mammary Carcinoma Eo771, Using C57 Black Mice.

| Injection of XYZ factor | | | | Data on tumor transplantation and growth | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Origin of XYZ | Stored Days | Stored Temp. | Days prior | Tumor and its origin | Host mice | Total mice | Grew tumor | $\Sigma_x$ | $M_x$ | $\Sigma_{x2}$ |
| | | | | A. Heterologous XYZ and Heterologous Host. | | | | | | |
| Eo771 57bl | 45 | 0°F | 34 | 15091a A | 57bl | 10 | 0 | 0.00 | 0.00 | 0.0 |
| Controls | | | | 15091a A | 57bl | 9 | 0 | 0.00 | 0.00 | 0.0 |
| | | | | B. Heterologous XYZ and Homologous Host. | | | | | | |
| Ba. Ca. C3H | 337 | 0°F | 16 | Eo771 57bl | 57bl | 10 | 9 | 23.70 | 2.37 | 108.5 |
| Controls | | | | | | 10 | 10 | 26.90 | 2.69 | 102.2 |
| Eo771 57bl | 45 | 0°F | 43 | 241-5 57bl | 57bl | 10 | 5 | | | |
| Eo771 57bl | 45 | 0°F | 43 | 241-5 57bl | 57bl | 8 | 2 | 15.42 | 0.86 | 56.5 |
| Controls | | | | 241-5 57bl | 57bl | 8 | 6 | | | |
| Controls | | | | 241-5 57bl | 57bl | 10 | 3 | 57.31 | 3.18 | 469.0 |
| E0771 57bl | 45 | 0°F | 50 | 241-16 57bl | 57bl | 8 | 4 | 10.14 | 1.27 | 85.0 |
| Controls | | | | 241-16 57bl | 57bl | 20 | 12 | 36.52 | 1.83 | 216.8 |
| 241-5 57bl | 68 | 0°F | 10 | Eo771 57bl | 57bl | 9 | 7 | | | |
| 241-5 57bl | 68 | 0°F | 10 | Eo771 57bl | 57bl | 9 | 8 | 65.36 | 4.36 | 450.3 |
| Controls | | | | Eo771 57bl | 57bl | 10 | 6 | | | |
| Controls | | | | Eo771 57bl | 57bl | 10 | 10 | 74.64 | 3.73 | 459.5 |
| 241-5 57bl | 68 | 0°F | 34 | Eo771 57bl | 57bl | 9 | 5 | 10.38 | 1.15 | 23.8 |
| Controls | | | | Eo771 57bl | 57bl | 9 | 8 | 17.18 | 1.91 | 58.4 |
| Summary XYZ injected animals | | | | | | 63 | 40 | 124.94 | 1.98 | 723.3 |
| Summary controls | | | | | | 77 | 55 | 212.65 | 2.76 | 892.0 |
| | | | | C. Homologous XYZ and Homologous Host. | | | | | | |
| Eo771 57bl | 45 | 0°F | 25 | Eo771 57bl | 57bl | 9 | 9 | | | |
| Eo771 57bl | 45 | 0°F | 25 | Eo771 57bl | 57bl | 10 | 10 | 135.37 | 7.12 | 1352 0 |
| Controls | | | | Eo771 57bl | 57bl | 10 | 6 | | | |
| Controls | | | | Eo771 57bl | 57bl | 8 | 8 | 52.86 | 2.94 | 302.4 |
| Eo771 57bl | 28 | 0°F | 18 | Eo771 57bl | 57bl | 10 | 10 | 63.44 | 6.34 | 472.5 |
| Eo771 57bl | 28 | 0°F | 19 | Eo771 57bl | 57bl | 10 | 9 | 4.98 | 0.50 | 3.9 |
| Controls | | | | Eo771 57bl | 57bl | 9 | 7 | 4.25 | 0.47 | 3.5 |
| Controls | | | | Eo771 57bl | 57bl | 8 | 8 | 25.72 | 3.22 | 110.1 |
| Summary XYZ injected animals | | | | | | 39 | 38 | 203.79 | 5.23 | 1828 |
| Summary controls | | | | | | 35 | 29 | 82.83 | 2.37 | 416 |

urement in each of the 12 experiments, the measurements used were the last series taken before the first animals of either test or control groups died from the tumor. The tumors employed were Eo771, 241-5, and 241-16, mouse mammary carcinomata, of C57 black origin; Barrett mammary carcinoma of C3H origin; and 15091a mammary carcinoma of Line A origin.† In each experiment Eo771 was used either as the source of the XYZ material or as the source of the tumor tissue, or both.

† The mouse tumors were obtained through the courtesy of various persons: Eo771 and 1509la from Dr. George D. Snell of Bar Harbor, Maine, 241-5 and 241-16 from Dr. Howard B. Andervont, Dr. Thelma Dunn and Dr. Harold L. Stewart, and the Barrett carcinoma from Dr. M. K. Barrett, and C57 black mammary carcinoma 755 from Dr. Edwin D. Murphy, all of Bethesda, Md.

Since Eo771, 241-5 and 241-16 originated in C57 black mice, such mice were considered "homologous hosts" when any of the 3 tumors was transplanted into them. On the other hand 15091a (of Line A origin) transplanted in C57 black mice, was said to have been inoculated into a heterologous host.

The XYZ material, if from a different tumor than that transplanted, was said to be heterologous, even though originating in the same anatomic site in the same inbred strain (*i.e.* 241-5, 241-16 and Eo771 in C57 black mice). Experiments with another mammary carcinoma No. 755 originating also in C57 black mice[19] are now in progress.

The XYZ material was prepared by placing fresh aseptically removed tumor tissue in

[19] Kaplan, Henry S., and Murphy, Edwin D., *J. Nat. Cancer Inst.*, 1949, **9**, 407.

the deep freeze chamber at 0°F and keeping it there for 28-337 days. In each instance a portion of the tumor tissue to be frozen was tested for viability by routine transfer. Each tumor for XYZ or for transplant was verified by histologic examination, using the blind check method for tumor identification. 241-5 and 241-16 were quite similar histologically and cage records were necessary to differentiate between them.

Since the Brown-Pearce and Bashford 63 XYZ factors withstand storage in the cold for at least a year and since a single injection leaves prolonged hypersusceptibility to the same tumor, some latitude was exercised in the storage (45-337 days) and interval before reinoculation (10-50 days) in the Eo771 experiments (Table I). The measurements of the transplanted tumor were made on the same day for both control and test mice, using calipers. The "volume" was the product of the caliper diameters in 3 dimensions. Although this introduced a considerable error when checked by weight of the tumors it gave a uniform value for both test and control animals.

The XYZ material and the tumor tissue for transplantation were prepared for inoculation by mincing and grinding in a mortar and diluting with normal saline (1 part tumor and 3 parts saline). In the last 2 experiments (Table I) Penicillin G sodium (30,000 units cc) buffered with sodium citrate (Lederle) was used as the diluent in the place of normal saline. 0.1 to 0.15 cc of the emulsion of the frozen XYZ or of the fresh tumor tissue as the case might be, was inoculated subcutaneously into the left groin.

A necropsy with histologic examination of lungs, heart, liver, and primary inoculation site and other selected tissue was made on each of the 250 mice. Because of the rapid growth of the tumors experiments in which the animals were transplanted with Eo771 were terminated at 15-30 days, whereas experiments with 241-5 and 241-16 were not terminated before 40-70 days depending on when the first animal died.

*Results.* The results are presented in tabular form under 3 categories, namely: A. Heterologous XYZ and heterologous host; B. Heterologous XYZ and homologous host; C. Homologous XYZ and homologous host (Table I). In no instance did a heterologous XYZ material produce an XYZ effect in either a heterologous or in a homologous host. (A and B, Table I). The summarized material indicates that 40 of the 63 heterologous XYZ injected animals (63%) had progressively enlarging tumor growths with an average size of 1.98 cc whereas 55 of the 77 control mice (71%) had progressively enlarging growths which averaged 2.76 cc (the difference $= 0.78 \pm 0.42$ cc; $t = 1.9$; $n = 138$; $P = 0.06$, not significant). This difference would indicate that the heterologous XYZ material produced a statistically insignificant inhibition of tumor growth. Likewise there was an insignificant inhibition of the incidence of transplanted tumors among the XYZ animals ($X^2 = 0.99$; $n = 1$; $P = 0.4$, not significant). The mean tumor size when the data for the Barrett carcinoma experiment was eliminated was 2.77 cc for 67 control mice as compared with 1.91 cc for 53 heterologous XYZ injected mice. The difference $0.86 \pm 0.45$ cc is suggestive of an inhibition.

In contrast to the above data the 39 animals injected with homologous XYZ into an homologous host had 38 progressively enlarging primary tumors (97%) as compared, with 29 progressively enlarging tumors among the 35 control mice in the 4 experiments (83%). The tumors averaged among the 39 XYZ injected animals 5.23 cc as compared with 2.37 cc among the 35 controls. The difference $2.86 \pm 0.84$ cc is statistically significant ($t = 3.4$; $n = 72$; $P = 0.01$—significant).

*Discussion.* In order to provide a basis for comparison with present results, the data of Snell, Cloudman, and Woodworth[17] pertaining to Eo771 has been abstracted and rearranged in the manner of the present experiments (Table II). This data includes observations on 213 inbred mice in which either Eo771 XYZ or Eo771 tumor transplants or both were used. There were three experiments with heterologous XYZ in heterologous hosts. The 26 XYZ injected animals had 17 progressively enlarging primary tumors (65%) as compared with 28 progressively en-

TABLE II.

Other Experiments with C57 Black Mouse Mammary Carcinoma Eo771. (Data abstracted and rearranged from the paper by Snell, Cloudman, and Woodworth[17] to correspond with the format in Table I).

| Injection of XYZ factor | | | | Data on tumor transplantation and growth | | | | |
|---|---|---|---|---|---|---|---|---|
| Origin of XYZ | Stored: Days | Stored: Temp. | Days prior | Tumor and its origin | Host mice | Total mice | Died, tumor | Maximum mean tumor size, sqcm |
| | | | A. Heterologous XYZ and heterologous host. | | | | | |
| Eo771 57bl | ? | lyoph. | 8-12 | C1498 57bl | 57ln | 6 | 4 | 5.53 |
| Eo771 57bl | ? | ,, | 8-12 | C1498 57bl | 57ln | 10 | 3 | 0.30 |
| L946 57bl | ? | ,, | 8-12 | Eo771 57bl | 57ln | 10 | 10 | 9.82 |
| Controls | | | | C1498 57bl | 57ln | 10 | 0 | 0.65 |
| ,, | | | | C1498 57bl | 57ln | 10 | 6 | 1.94 |
| ,, | | | | C1498 57bl | 57ln | 10 | 7 | ? |
| ,, | | | | C1498 57bl | 57ln | 10 | 7 | ? |
| ,, | | | | Eo771 57bl | 57ln | 8 | 8 | 12.99 |
| Summary XYZ injected animals | | | | | | 26 | 17 | 5.17 |
| Summary controls | | | | | | 48 | 28 | 4.64 |
| | | | B. Heterologous XYZ and homologous host (no experiments reported). | | | | | |
| | | | C. Homologous XYZ and homologous host (no experiments reported). | | | | | |
| | | | D. Homologous XYZ and heterologous host. | | | | | |
| Eo771 57bl | ? | lyoph. | 8-12 | Eo771 57bl | 57ln | 10 | 8 | 3.26 |
| Eo771 57bl | ? | ,, | 8-12 | Eo771 57bl | 57bred | 10 | 0 | 0.25 |
| Eo771 57bl | ? | ,, | 15 | Eo771 57bl | C58 | 15 | 15 | 4.97 |
| Eo771 57bl | ? | ,, | 8-12 | Eo771 57bl | C58 | 10 | 7 | 2.31 |
| Eo771 57bl | ? | ,, | 8-12 | Eo771 57bl | BAlbC | 12 | 3 | 0.17 |
| Eo771 57bl | ? | ,, | 8-12 | Eo771 57bl | BAlbC | 12 | 8 | 5.94 |
| Eo771 57bl | ? | ,, | 8-12 | Eo771 57bl | BAlbC | 9 | 7 | 3.90 |
| Controls | | | | Eo771 57bl | 57ln | 5 | 3 | 4.25 |
| ,, | | | | Eo771 57bl | 57bred | 10 | 0 | 0.13 |
| ,, | | | | Eo771 57bl | C58 | 14 | 2 | 0.24 |
| ,, | | | | Eo771 57bl | C58 | 10 | 0 | 0.13 |
| ,, | | | | Eo771 57bl | BAlbC | 12 | 0 | 0.42 |
| ,, | | | | Eo771 57bl | BAlbC | 10 | 0 | 0.44 |
| Summary XYZ injected animals | | | | | | 78 | 48 | 3.09 |
| Summary controls | | | | | | 61 | 5 | 0.60 |

larging tumors among 48 controls (58%). This difference in incidence was not significant ($X^2 = 0.356$; n = 1; P = 0.6, not significant). The average size of the tumors in the 26 heterologous XYZ injected mice was 5.17 sq cm as compared with 4.64 sq cm among the 48 controls. The difference 0.53 $\pm$ 0.47 sq cm was not statistically significant.

When the data for heterologous XYZ injected animals of Tables I and II with their respective controls were combined there were 57 progressively enlarging tumors among 99 heterologous XYZ animals (58%), and 83 progressively enlarging tumors among 134 controls (62%). The difference was not statistically significant. The mean size of the tumors in the two groups was almost identical and the data of Snell, Cloudman and Woodworth and ours are in entire agreement. To us the data indicates that heterologous XYZ material has no appreciable effect on transplanted tumor growth in either homologous or heterologous hosts.

In 7 experiments 48 of the 78 mice injected with homologous XYZ material by Snell, Cloudman, and Woodworth had progressively enlarging tumors (62%) as compared with 5 tumors among 61 control mice (8%). This difference was statistically significant ($X^2 = 40.4$; n = 1; P = 0.0001—significant). The tumors among the 78 experimental mice averaged 3.09 sq cm and among 61 controls 0.60 sq cm. The difference 2.49 $\pm$ 0.34 sq cm was statistically significant (t = 4.3; P = 0.0001—significant). The key to the puzzle would seem to be the use of homologous XYZ material, and with such material heterologous inbred hosts were not always resistant to tumors of foreign strain origin.

In the experiments summarized in Tables I and II cross XYZ reactions were studied among 5 neoplasms of C57 black origin, and

the reactions in a sixth are now being tested (mammary carcinoma 755). Four of the 6 are mammary carcinomata of C57 black origin. (15091 of Line A origin, and Barrett carcinoma of C3H origin are also mammary carcinomata). If the XYZ material be of cellular origin it must be a permanent chemical mutation not present in the other mammary tissues and tumors of the same inbred strain. It must be perpetuated ad infinitum in the specific cells, but in no other and must exert a powerful influence on the continued transplantabiltiy and growth of the specific cells.

If the XYZ material be an inert contaminating virus[20] (a possibility which was considered early in the work)[14] it might conceivably act as follows: The injected XYZ material immunizes the host to the virus and the subsequently transplanted tumor grows with untrammeled or natural vigor, unimpeded by its contaminating virus.[21,25] This possibility was considered with the Brown-Pearce rabbit tumor XYZ material. A Lilac rabbit (resistant strain), in which a Brown-Pearce transplant had grown subcutaneously, regressed and disappeared, was sacrificed. Blood serum from this "immune" rabbit was mixed in equal parts with XYZ material and allowed to stand for some hours in a kitchen type ice box. This immune serum-XYZ mixture injected prior to tumor transplantation enhanced the XYZ effect. If an inert contaminating virus were present, then, the virus-serum mixture perhaps should have lessened the XYZ effect instead of increasing it. Also probably every strain of Brown-Pearce tumor throughout the world today was derived from animals which had had XYZ material before tumor inoculation and shipped (by AEC) to various places. Our own strains came from several generations of such XYZ animals yet the XYZ material may be obtained with regularity and ease from the frozen tissue. If a virus, it is inert in the sense that no fatalities or clinical disease follows its injection, and dynamic in the sense that metastases and local growth of the homologous tumor are enhanced by its injection. The recent experimental work of Kaplan and Murphy on insufficient radiation inducing more metastases is very suggestive of the XYZ effect.[19] The Brown-Pearce material is almost equally as effective when given 2 weeks after tumor transplantation as 2 weeks before.[1] The XYZ effect does not result when Berkefeld or Seitz filtrates of fresh tumor[22,23] or nuclei and cytoplasm of fresh tumor[24] are injected prior to tumor transplantation. A single injection of the XYZ factor into immune rabbits which had been tested and retested by inoculations of Brown-Pearce tumor broke down the resistance of 33% of the animals upon subsequent challenge with fresh tumor tissue.[1]

*Summary and conclusions.* 1. Twelve experiments were made with mouse mammary carcinoma Eo771 (C57 black origin) using 250 C57 black mice. Complementary use was made of mouse mammary carcinomata 241-5, 241-16 (C57 black origin), 15091a (Line A origin), and Barrett (C3H origin).

2. A highly selective XYZ factor (entirely comparable with those described for the Brown-Pearce rabbit tumor and Bashford mouse mammary carcinoma 63), resulting in enhanced tumor growth, was found to be uniformly present in the frozen Eo771 tumor tissue. The XYZ effect was present when the XYZ factor was derived from the homologous tumor and absent when derived from a heterologous tumor, even from other mammary carcinomata of the same highly inbred C57 black strain. This high tissue selectivity of the XYZ factor was not limited or determined by the host into which the tumor cells were transplanted. The factor was equally effective in homologous or heterologous transplant-

20 Taylor, M. J., and MacDowell, E. C., *Cancer Research*, 1949, **9**, 144.

21 Kritzler, Robert A., Mulliken, B., and Turner, Joseph C., *Cancer Research*, 1949, **9**, 74.

22 Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 111.

23 Drysdale, George R., and Casey, Albert E., unpublished experiments.

24 Skipper, Howard, Edwards, P. C., Drysdale, George R., and Casey, Albert E., unpublished experiments.

25 Bang, F. B., and Gey, G. O., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 78.

ed hosts, so long as the tumor cells were viable in the foreign host.

3. XYZ factors have thus far been proven for only 4 transplanted neoplasms of mice and rabbits all of which are Grade III-IV by histologic grading. No such factors have been observed in adult tissues, leukemias, benign and low grade tumors.

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## *In vitro* Determination of Bacterial Sensitivity to Aureomycin. (17340)

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Results of aureomycin sensitivity studies of various microorganisms have deviated greatly from one another as reported by different laboratories. This has been best exemplified by wide divergences in the reported sensitivities of staphylococci to this agent. Paine and associates[1] employing both tube dilution and plate methods, noted that 22 strains of *S. aureus* required 1 to 2 μg of aureomycin per ml for inhibition. The 5 remaining strains were inhibited by higher concentrations of the antibiotic, ranging up to 12.5 μg per ml. The type of media used was not described. Price and collaborators[2] reported *S. aureus* to be inhibited by a range of .09 to 25 μg of aureomycin per ml. The determinations were made following incubation of the organisms in penicillin assay broth for 24 hours. Bryer and associates[3] stated that 0.6 μg of aureomycin per ml inhibited staphylococci. Their report contained no statement about the method of determination. Twenty-one strains of this group of organisms tested by Lankford and Lacy[4] were inhibited by .05 to 0.2 μg of aureomycin per ml. Bacto-tryptose agar was the medium employed. With a tryptose broth turbidimetric method and 50% inhibition of growth in 18 hours as the end-point, the most sensitive strain was inhibited by .012 μg of aureomycin per ml and the most resistant by .033 μg per ml.

This study is an investigation of possible causes for discrepancies in the results obtained by others. In addition, a search has been made for a simple and reliable method of determining bacterial sensitivity to aureomycin.

*Methods.* Growth curves of *S. aureus,* enterococcus, *S. panama,* and *E. coli* were determined by a turbidimetric method. 0.1 ml inocula of 18 hour broth cultures were added to 18 mm test tubes containing 10 ml of tryptose broth (Difco). Aureomycin crystals were dissolved in cold sterile distilled water, diluted in tryptose broth, and added to the system immediately.* The final concentrations were .01, .02, .05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg per ml. A control tube without aureomycin was included in each determination. The cultures were incubated at 37°C and the turbidity was measured in a Coleman Junior spectophotometer with a 650 m filter. Values of less than 0.1 unit of optical density indicated marked bacteriostasis and were chosen as end-points.

Tryptone glucose extract agar with para-aminobenzoic acid (Difco) was the medium used for the plate method. Aureomycin was incorporated in this medium in final concentrations identical with those described above plus 20 and 50 μg per ml, and 18 hour broth cultures were streaked on the plates. Readings were made at various intervals of time,

1 Paine, T. F., Collins, H. S., and Finland, M., *J. Bact.,* 1948, **56**, 489.

2 Price, C. W., Randall, W. A., and Welch, H., *Ann. New York Acad. Sci.,* 1948, **51**, 211.

3 Bryer, M. S., Schoenbach, E. B., Chandler, C. A., Bliss, E. A., and Long, P. H., *J.A.M.A.,* 1948, **138**, 117.

4 Lankford, C. E., and Lacy, H., *Texas Rep. Biol. Med.,* 1949, **7**, 111.

* Solutions of aureomycin in water retained full potency if stored in the frozen state.

TABLE I.
Turbidimetric Method. (Concentration of aureomycin in μg per ml).

| | Tryptose broth—hr | | | | | | Tryptose broth plus 10% serum—hr | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | 14 | 20 | 40 | 50 | 60 | 4 | 14 | 20 | 40 | 50 | 60 |
| *S. aureus* (41552) | .02–.05 | .1–.2 | .2–.5 | .5 | — | 1–5 | .05 | .2–.5 | .5 | — | — | 5 |
| *S. aureus* (37215) | .05 | 2 | — | — | .5 | — | .05 | .5 | — | — | 5 | — |
| Enterococcus (40056) | .1 | — | .5 | — | 5 | — | .2 | — | 2 | — | 10 | — |
| *Sal. panama* (40615) | .5 | 1–2 | 2–5 | 10 | — | — | .5 | 2 | 5 | 10 or more | | |
| *E. coli* (41010) | .5 | 1 | 2 | — | 10 | — | .5–1 | 1 | 5 | — | — | — |

The figures represent the concentration of aureomycin in μg per ml, required to inhibit growth.

complete inhibition of growth being the end-point.

A number of sensitivity determinations were made with pooled sterile human sera or whole horse blood added to the basal media. Two percent hemolyzed horse blood was used for the turbidimetric method. The blood added to tryptone glucose extract agar invariably hemolyzed.

The one precaution to be consistently observed is rapid preparation and inoculation of the aureomycin media. The whole task should be performed within a few hours due to rapid deterioration of this antibiotic.

*Results.* Inhibitory levels of aureomycin for 2 strains of *S. aureus* as determined by the turbidimetric method were .02 to .05 μg per ml after 4 hours of incubation, 0.1 to 0.2 μg per ml after 14 hours, 0.2 to 0.5 μg per ml after 20 hours, 0.5 μg per ml in 40 to 50 hours, and 1 to 5 μg per ml after 60 hours (See Table I). A composite growth curve derived from 9 experiments is presented in Fig. 1. It demonstrates partial inhibition for *S. aureus* by .01 and .02 μg of aureomycin per ml, complete inhibition for 7 hours by .05 μg per ml, and failure of this and higher concentrations to inhibit bacterial growth as the period of incubation is lengthened. One strain of enterococcus was inhibited by 0.1 μg per ml at 4 hours, 0.5 μg per ml at 20 hours, and 5 μg per ml at 50 hours. *Salmonella panama* was inhibited by 0.5 μg per ml at 4 hours, 1 to 2 μg per ml at 14 hours, 2 to 5 μg per ml at 20 hours, and 10 μg per ml at 40 hours. For one strain of *E. coli,* the inhibitory levels were 0.5 μg per ml at 4 hours, 1 μg per ml at 14 hours, 2 μg per ml at 20 hours, and 10 μg per ml at 50 hours. With serum present in 10% concentration, the levels were the same or somewhat higher. Increases were usually to the extent of one tube dilution, but were occasionally 5 to 10-fold (3 to 4 tube dilutions) following long periods of incubation.

Subculture of the media in the tubes containing 10 μg of aureomycin per ml invariably revealed a few viable organisms. *S. aureus* so isolated was inhibited by .02 μg of aureomycin per ml at 4 hours and 0.1 μg per ml at 20 hours. For *Sal. panama,* the values were 0.5 μg per ml at 4 hours and 2 μg per ml at 14 hours. These values were comparable to those obtained with the parent strains.

Aureomycin incubated for 18 hours at 37°C was tested against *S. aureus* and *Sal. panama.* The inhibitory levels for *S. aureus* were 0.2 μg per ml at 4 hours and 1 μg per ml at 20 hours. Pre-incubation levels were .02 μg per ml and 0.2 μg per ml at 4 and 20 hours respectively. Two micrograms per ml at 4 hours and 5 to 10 μg per ml at 20 hours were required by *Sal. panama* as compared with control levels of 0.5 μg per ml at 4 hours and 2 μg per ml at 20 hours. This represents about 60 to 90% deterioration of aureomycin in 18 hours.

The aureomycin inhibitory levels observed following incubation of *S. aureus* and *Sal. panama* for 4 hours in tryptose broth containing 2% horse blood were .05 μg and 0.5 μg per ml respectively. These values corresponded closely to those obtained with the simple tryptose broth medium.

Aureomycin was added to cultures of *S. aureus, E. coli,* and enterococcus one or 2 hours after growth commenced. Further growth was inhibited if aureomycin levels of

PLATE II.
Plate Method.

| Hours | Tryptone glucose extract agar-PABA | | | | | Tryptone glucose extract agar-PABA Serum | | | | | Tryptone glucose extract agar-PABA Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12 | 18 | 24 | 36 | 60 | 12 | 18 | 24 | 36 | 60 | 12 | 18 | 24 | 36 | 60 |
| *S. aureus* (41552) | .05–.2 | .1–.5 | .5 | .5 | 1–20 | .5–1 | 1–2 | 2 | 5–10 | 10–50 | .5–1 | 1–2 | 2 | 5 | 20 |
| *S. aureus* (42044) | .05–.1 | .1–.5 | .2–.5 | .5–1 | 1–2 | .5–1 | 1–2 | 2 | 5 | 5–20 | .5–1 | 2 | 2 | 5 | 20 |
| Enterococcus (44962) | .1–.5 | .5 | .5–1 | 1–2 | 10–20 | .5–1 | 1–2 | 2 | 5–10 | 10–over 50 | .5–1 | 1–2 | 2–5 | 10 | 20–50 |
| *Sal. panama* (40615) | 1–2 | 1–2 | 2–5 | 5–10 | 10–20 | 2–5 | 2–10 | 10 | 5–20 | 10–over 50 | 5–10 | 10 | 10–20 | 20–50 | 50–over 50 |
| *E. coli* (41010) | 1–2 | 2 | 5 | 5–20 | 5–50 | 2 | 2–10 | 10 | 5–over 50 | 10–over 50 | 2–5 | 5 | 10 | 50 | Over 50 |
| *S. aureus* (37215) | .05–.2 | .1–.2 | .1–.2 | | .1–.2 | | | | | | | | | | |
| Enterococcus (40056) | .1–.2 | .2–1 | | .5–1 | 1–2 | | | | | | .5–1 | 1–2 | 2–5 | 10 | 20–50 |

The figures represent the concentrations of aureomycin in μg per ml, required to inhibit growth.

the magnitude described above as inhibitory were present. No lysis was evident even with concentrations of the agent one hundred times greater than those sufficient for bacteriostasis.

The common ranges of aureomycin sensitivity determined by the plate method for 6 strains of *S. aureus* were .05 to 0.2 μg per ml at 12 hours, 0.1 to 0.5 μg per ml at 18 hours, 0.5 to 1 μg per ml at 24 hours, 0.5 to 5 μg per ml at 36 hours, and 1 to 20 μg per ml at 60 hours (See Tables II and III). The values for 5 strains of enterococcus were 0.1 to 0.5 μg per ml at 12 hours, 0.2 to 1 μg per ml at 18 hours, 0.5 to 1 μg per ml at 24 hours, 0.5 to 2 μg per ml at 36 hours, and 1 to 20 μg per ml at 60 hours. Aureomycin inhibitory levels for *Sal. panama* were 1 to 2 μg per ml at 12 and 18 hours, 2 to 5 μg per ml at 24 hours, 5 to 10 μg per ml at 36 hours, and 10 to 20 μg per ml at 60 hours. The *E. coli* values were about the same as those for *Sal. panama* Considerably higher levels were required for inhibition when serum or blood was present. At 18 hours, for example, these ranged from 2 to 20 times greater for the gram positive cocci, and 1 to 10 times increase in aureomycin concentration for the gram negative bacilli.

Four strains of *S. aureus* had been tested against penicillin and streptomycin. Three were penicillin-resistant and one was streptomycin-resistant. All strains were equally sensitive to aureomycin.

*Discussion.* The data presented in this study are in close agreement with those of Lankford and Lacy.[4] Very low concentrations of aureomycin inhibited *S. aureus* if the medium used was plain tryptose broth or tryptone glucose extract agar. The same was true of other organisms tested. Lowest values were obtained with the 4 hour turbidimetric method. Addition of serum or blood to the medium increased the concentration of aureomycin required for inhibition, especially when incubation was prolonged. This effect was quite marked with the plate method. The absence of bactericidal action by aureomycin and the rapid deterioration of this agent during incubation offer an adequate explanation of these observations. A richer medium would be expected to accelerate bacterial growth

TABLE III.
Aureomycin Sensitivities in μg per ml. (Tryptone glucose extract agar plate method).

| Organism | Hr | | | |
|---|---|---|---|---|
| | 12 | 18 | 24 | 36 |
| *S. aureus* (Strains 1, 44488, 44381) | 0.2 | 0.5 | 0.5 | 5 |
| Enterococcus (40156) | 0.1 | — | — | 1 |
| Enterococcus (43284) | 0.1 | 0.2 | — | — |
| Enterococcus (45145) | 0.2 | 0.2 | 0.5 | — |
| *E. coli* (40382) | 1 | 1–2 | — | 5 |
| *E. coli* (44532) | 1 | 5 | 5 | 20 |
| *E. coli* (43768) | 1 | 2 | 5 | 20 |
| *Ps. aeruginosa* (43919) | 50 | 50 | 50 | Over 50 |

more rapidly as the inhibitory action of the drug declined. Therefore, the higher values reported by other investigators may be ascribed to their use of highly nutritive media, probably containing serum or blood, and to incubation for prolonged periods of time. The supposed direct inactivation of aureomycin by serum or blood reported by Chandler and Bliss[5] may have reflected an interplay of these factors rather than specific action on the drug.

The turbidimetric method with tryptose broth as the medium is probably the most accurate. It has the advantage of permitting rapid determinations, 3 or 4 hours of incubation being sufficient. It is more tedious and exacting than a simple plate method using tryptone agar, which may be preferable for routine laboratory use. Values obtained will be higher than those obtained by the turbidimetric method. Readings should be made within 12 hours of inoculation if possible since the error will be diminished with shorter periods of incubation. A correction factor may be employed, but this will vary widely with the experimental situation even at 12 hours. Eighteen hour determinations may be as much as 25 times greater than the equivalent 4 hour turbidimetric value.

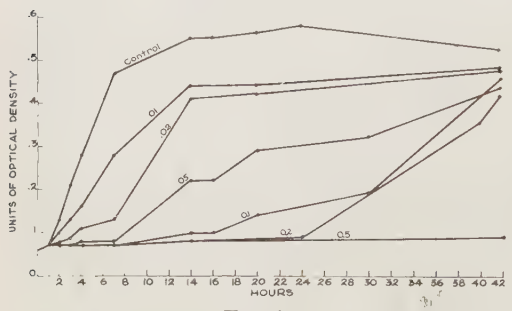


FIG. 1.
*Staph. aureus* growth curves.
(Aureomycin concentration in micrograms per ml).

Noting that bacterial sensitivity decreased as the size of the inoculum was increased, Harrell and associates[6] have warned of the potential danger of aureomycin resistance. This possibility has received further support from the detection of viable bacteria in the presence of very high concentrations of the agent. Such organisms were found on subsequent test to be fully sensitive as studied in this laboratory with the turbidimetric method. It is suggested that these bacteria were "persisters."

*Summary.* 1. Gram positive cocci and gram negative bacilli were tested turbidimetrically for aureomycin sensitivity. The inhibitory levels were .02 to .05 μg per ml for *S. aureus*, 0.1 μg per ml for enterococcus, and 0.5 μg per ml for *Sal. panama* and *E. coli*.

2. Aureomycin was not inhibited by the presence of blood or serum in the medium.

3. Increased length of incubation and addition of serum or blood to the medium resulted in higher levels of inhibition, particularly with the plate method. An explanation of this effect is offered.

4. Tryptone glucose extract agar was used for a plate method. Values were higher and less consistent than with the turbidimetric technic

5 Chandler, C. A., and Bliss, E. A., *Ann. New York Acad. Sci.*, 1948, **51**, 221.

6 Harrell, G. T., Meads, M., and Stevens, K., *South. Med. J.*, 1949, **42**, 4.

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# Effect of Androgen on Concentration of Certain Amino Acids in the Rat Prostate.* (17341)

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Although recent reports[1] have appeared describing the effects of hormones on the biochemical constituents of organs stimulated by those hormones, no information is available relative to the influence of hormones on the free amino acids of such tissues. As part of a large program of growth investigation, it became of interest to make such a study.

Young, adult male rats (140-160 g) of the Sprague-Dawley strain were used. Orchidectomy was performed through a mid-ventral incision with the rats under light ether anesthesia. A period of 15 days was allowed for prostatic atrophy. The castrated rats which received androgen were given 0.5 mg of testosterone propionate (Oreton†) in 0.25 ml of corn oil subcutaneously each day for periods of 1, 4, 8, and 12 days. All animals were fasted 24 hours, killed by decapitation, and the ventral lobes of the prostates were removed. A sufficient number of glands was pooled to furnish a total of 300-500 mg of tissue. The tissues were prepared and analyzed by paper chromatography according to the method of Awapara.[2] Qualitative separation of the various amino acids was done by the two-dimensional method, and one-dimensional quantitative determinations were carried out for alanine, glycine, glutamic acid, and aspartic acid. The results of the former procedure are shown in Fig. 1, and the values obtained by the latter method are given in Table I.

It can be seen that following castration there are fewer amino acids determinable by the qualitative technic, and with replacement therapy the variety progressively approaches the normal. This can be interpreted in at least two ways: a) there is an absolute disappearance of certain amino acids, and b) the concentration of these amino acids is reduced below the sensitivity of the method. The latter explanation seems to be the more reasonable. Quantitation of the 4 principal amino acids shows that castration is followed by a marked reduction in their concentrations. It is apparent also that 8-12 days of replacement therapy with 0.5 mg of testosterone propionate each day is necessary before the amino acid concentrations approximate the normal levels. These data cannot be compared with the findings of the authors previously quoted because in those experiments the injections of androgen were begun on the day of castration and, therefore, no time was allowed for atrophy.

In Fig. 1 it can be seen that the normal prostate has at least 4 peptides (14 A, B, C, D) not seen in the glands of castrates or castrates receiving androgen. It is also of interest to note that the prostate of the castrate animal has a substance, probably a peptide, which disappears after 4 days of treatment with androgen. The physiologic significance of these peptides is not at all clear and must await further study. An unidentified substance (Fig. 1, No. 15) was found to be present in every case. Preliminary studies in this laboratory suggest that this substance is cystine.

Androgen replacement stimulates growth and renews the secretory activity of atrophic prostate glands. Since these changes are concomitant with a progressive increase in the concentration of free amino acids, it may be concluded that growth is associated with an increased propensity of tissue for concentrating and retaining these substances. This concept would appear to be important in

* This work was supported in part by a grant (No. INSTR 23) from the American Cancer Society.

1 Davis, J. S., Meyer, R. K., and McShan, W. H., *Endocrinology*, 1949, **44**, 1.

† The Oreton used was supplied by the Schering Corporation through the courtesy of Dr. Irwin Schwenk.

2 Awapara, J., *J. Biol. Chem.*, 1949, **178**, 113.

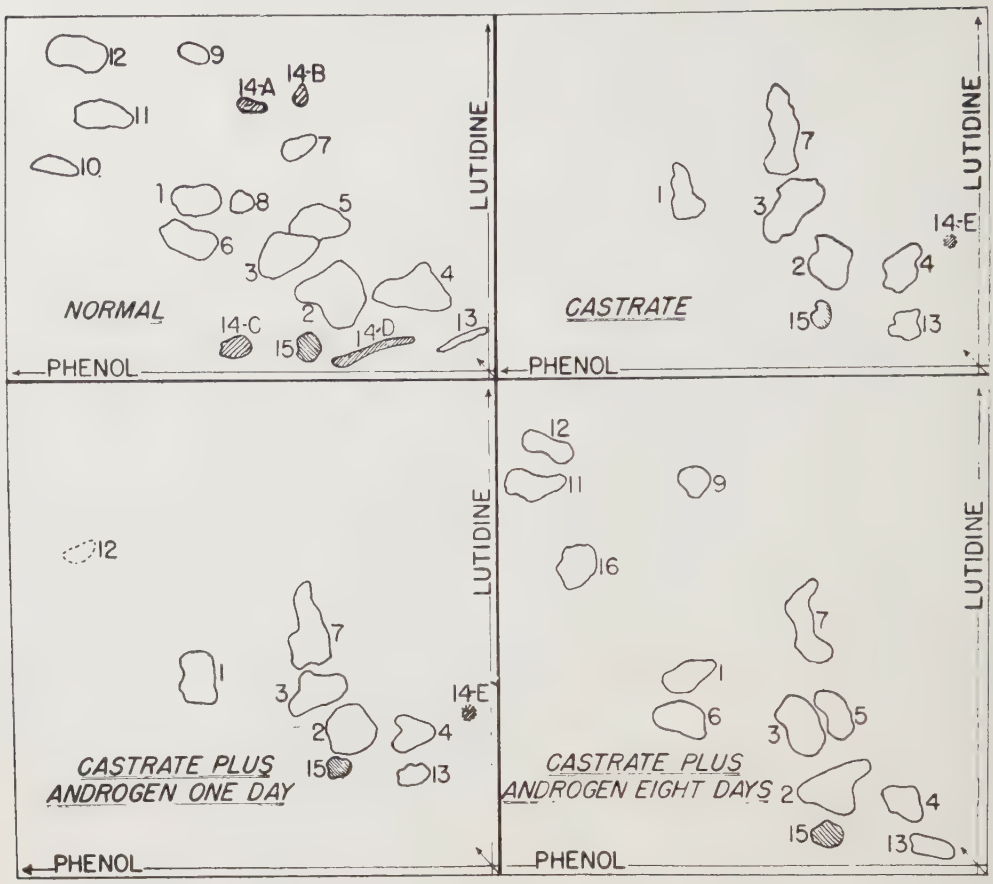


FIG. 1.

Chart showing the distribution of free amino acids as demonstrated by two-dimensional paper chromatography. The numbered areas indicate the positions of the following substances: 1. alanine; 2. glutamic acid; 3, glycine; 4. aspartic acid; 5. serine; 6. glutamine; 7. taurine; 8. threonine; 9. tyrosine; 10. proline; 11. leucine; 12. phenylalanine; 13. glutathione; 14. A, B, C, D, E, unidentified peptides; 15. unidentified (cystine ?); 16. valine.

understanding the stepwise mechanism of protein synthesis in growth. One might question the contribution of prostatic secretion, *per se,* to the increase in amino acid levels. Unpublished data in this laboratory obviate this possibility, in that no free amino acids have been demonstrated in prostatic fluid by the same procedure used above.

TABLE I.
Effect of Castration and Androgen Replacement on Amino Acids in Rat Prostate.

| Treatment | Prostate wt,* mg | Amino acid concentration mg/100 g tissue | | | |
|---|---|---|---|---|---|
| | | Aspartic acid | Glutamic acid | Glycine | Alanine |
| Castrate—15 days | 45 (9) | 12 | 16 | 9 | — |
| Castrate + androgen 24 hr | 55 (6) | 13 | 20 | 12 | — |
| Castrate + androgen 4 days | 178 (4) | 12 | 29 | 16 | 9 |
| Castrate + androgen 8 days | 562 (2) | 23 | 57 | 25 | 43 |
| Castrate + androgen 12 days | 762 (2) | 44 | 100 | 44 | 36 |
| Normal | 700 (2) | 58 | 102 | 45 | 40 |

* The figures in parentheses are the numbers of glands from which the mean weight given was obtained.

*Summary.* A decrease in the amounts and numbers of free amino acids determinable by paper chromatography is associated with prostatic atrophy after castration. Replacement therapy in the castrated animal is followed by a progressive increase toward normal. The relation of these findings to growth processes is briefly discussed.

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## The Electroencephalogram in Parkinsonism. (17342)

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It is generally recognized that the pathological process in Parkinsonism centers about the basal ganglia. The electrical activity of this region was studied by Meyers and Hayne[1] by direct insertion of insulated electrodes. They found normals to have a striatal rhythm faster than that of the cortex, while patients with Parkinsonism showed sequences of 25 cycle rhythm not recorded from the cortex. Spiegel[2] found the potentials from the cat's thalamus to be much like those from the cortex. Using an electrode driven into the sphenoidal bone Grinker and Serota[3] found 4 to 6 cycle waves as well as alpha rhythm. Using nasopharyngeal electrodes, Barnett[4] failed to find any 4 cycle activity.

Using conventional scalp electrodes, Yeager and Baldes[5] reported 4 cycle waves not synchronous with the tremor in patients with Parkinsonism. Jasper and Andrews[6] found no slow waves in two patients with unilateral Parkinsonism, but did record such activity from 2 patients with advanced bilateral disease. They concluded that the slow activity was present in cortical leads only when tremor was bilateral. Schwab and Cobb[7] recorded similar potentials from patients with Parkinsonism, but were of the opinion that the slow waves were artifacts from head movement, since they were able to suppress them by mechanically restraining the head. In view of these conflicting opinions, it seemed worth while to investigate the problem further.

The basal leads which we employed consisted of silver cannulae, insulated save at the tip with bakelite varnish, which were inserted, after topical cocainization, through the nostrils and into the sphenoidal sinuses, where the tips lay against the posterior wall. X-ray examination showed the tips to be in close proximity to the floor of the sella turcica in the majority of cases. In addition to the two sphenoidal leads, scalp electrodes of hypodermic needle type[8] were placed on each side in the frontal, central, and occipital regions, and the two mastoid regions were grounded. The potentials were amplified by a Grass 4 channel electroencephalograph. Recordings were made between the two sphenoidal electrodes, as well as between the sphenoidal electrodes and the pairs of scalp electrodes. Unipolar recordings from the sphenoidal and scalp electrodes were also made.

In general, where both sphenoidal electrodes made good contact, which occurred about half the time, the intersphenoidal potentials resembled most those recorded from the central scalp electrodes, showing mostly 20 cycle ac-

1 Meyers, Russell, and Hayne, Robert, *Trans. Am. Neurol. Assn.*, 1948, p. 10.

2 Spiegel, E. A., *Am. J. Physiol.*, 1937, **118**, 569.

3 Grinker, R. R., and Serota, H., *J. Neurophysiol.*, 1938, **1**, 573.

4 Barnett, A., *J. Lab. and Clin. Med.*, 1941, **26**, 1659.

5 Yeager, E. L., and Baldes, E. J., *Proc. Staff Meet. Mayo Cl.*, 1937, **12**, 705.

6 Jasper, H. H., and Andrew, H. L., *J. Neurophysiol.*, 1938, **1**, 87.

7 Schwab, R., and Cobb, S., *J. Neurophysiol.*, 1939, **2**, 36.

8 Newman, H. W., *Stanford Med. Bull.*, 1949, **7**, 61.

tivity of low voltage, with some 10 cycle waves. The potentials recorded between the sphenoidal electrodes and the scalp electrodes corresponded to those secured from the corresponding scalp electrode and ground. Thus there were no waves felt to be characteristic of the sphenoidal leads.

Records were secured from 12 patients with Parkinsonism. Of these, 6 were incidental to cerebral arteriosclerosis and 6 were post-encephalitic. Nine showed gross tremor, bilateral in all cases. Of these patients with tremor, 5 showed no evidence of slow activity on the EEG, either from the sphenoidal or the scalp leads. The remaining 4 showed slow activity, all of them from the sphenoidal leads, and 3 from one or more scalp leads as well. Fig. 1 shows the 4 cycle activity recorded from the sphenoidal and frontal leads in one of these patients, and its disappearance on opening the eyes, indicating that it was due to eye movement incidental to the tremor. Fig. 2 shows 4 cycle activity in another patient from the sphenoidal and occipital leads, and its disappearance when the patient's hands were restrained.

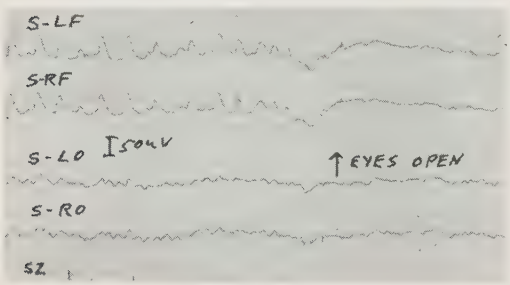


FIG. 1.
Disappearance of 4 cycle activity on opening the eyes in a patient with Parkinsonism. The electrode placement is S-LF, sphenoidal to left frontal; S-RF, sphenoidal to right frontal; S-LO, sphenoidal to left occipital; S-RO, sphenoidal to right occipital.

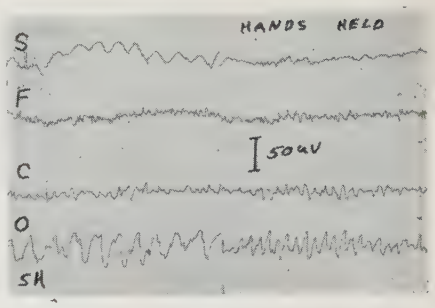


FIG. 2.
Disappearance of 4 cycle rhythm on mechanical restraint of the hands in a patient with Parkinsonism. The electrode placement is S, sphenoidal; F, frontal; C, central, and O, occipital, all bipolar.

In the one patient who showed slow activity from the sphenoidal leads but not from the scalp leads it is a little more difficult to dismiss the slow rhythm as artifact, but since it too was suppressed by mechanical restraint it seems reasonable to ascribe it to head movement.

In none of the 3 patients who had no gross tremor was any slow activity demonstrated from either the sphenoidal or scalp leads.

*Summary.* Patients with Parkinsonism do not show slow activity in the EEG, using either sphenoidal or scalp electrodes, except that incidental to movement artifact.

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## Blood Level of Sodium l-Methyl Butyl Barbituric Acid and Duration of Anesthesia in Rabbits. (17343)

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Since the report of Koppanyi, *et al.*,[1] on the barbiturate-cobalt reaction extensive investigations of the metabolic fate of the barbiturates have taken place. The recent development of an ultraviolet spectrophotometric method utilizing small samples of blood has

[1] Koppanyi, T., Murphy, W. S., and Krop, S., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 542.

TABLE I.
Blood Level (mg/%) at Time of Awakening as Influenced by Dose and Route of Administration.

| Intravenous, 30 mg/kg | Intramuscular, 35 mg/kg | Intraperitoneal, 35 mg/kg | Subcutaneous, 35 mg/kg | Oral, 50 mg/kg |
|---|---|---|---|---|
| 1.2 | 1.4 | 1.2 | 0.9 | 1.1 |
| 1.1 | 1.2 | 1.2 | 1.6 | 1.0 |
| 1.4 | 1.1 | 0.9 | 1.4 | 1.1 |
| 1.4 | 1.7 | 1.2 | 1.0 | 1.1 |
| 1.1 | 1.1 | 1.1 | 1.6 | 1.1 |
| 1.0 | 1.0 | 1.4 | 1.4 | 1.7 |
| *1.20 | 1.25 | 1.17 | 1.32 | 1.12 |
| † .17 | .16 | .30 | .30 | .26 |

* Mean.
† Standard deviation.

made feasible the reinvestigation of the variations in the blood level of the barbiturate during anesthesia. This report describes the relationship found between the blood level of the barbiturate and the duration of anesthesia.

The sodium salt of 1-methyl butyl barbituric acid was used throughout the study. Female albino rabbits weighing about 2 kg were given a single dose of the drug after having been allowed only water for the 12 to 18 hours preceding injection. Blood samples of 5 cc were obtained by cardiac tap and analyzed by the ultraviolet spectrophotometric method of Goldbaum.[2] Each figure in Table I represents the average of a duplicate analysis. The "awakening time" was taken the instant the animal spontaneously assumes the prone position.

A group of 19 animals was given a dose of 35 mg/kg of sodium pentobarbital intravenously. The injections were made in the marginal vein at the rate of 1 cc (20 mg pentobarbital/cc) per minute. Following the injection the animals were placed in the supine position. Blood samples were withdrawn both at 10 minutes and upon spontaneous righting.

Despite careful attention to the technic of injection there was a wide variation in the level of the drug found in the circulating blood ten minutes after injection. A mean of 3.21 ± 0.22 mg % with a range of from 2.3 to 3.9 mg % was found. In contrast, the level on awakening was constant exhibiting a mean of 1.16 ± 0.04 mg %. The blood level at the time of awakening was found to be unrelated to the duration of anesthesia. Thus, the shortest time recorded, 45 minutes, was found in an animal with a level on awakening of 1.2 mg %; whereas the animal which slept longest, 225 minutes, had an awakening level of 1.0 mg %. Upon statistical evaluation the correlation coefficient between duration of anesthesia and awakening blood level is found to be $r = -0.181$, indicating no dependence. Neither could a relation between the total sleeping time and the concentration of drug in the blood at 10 minutes be established. Although those animals which slept over 200 minutes had 10 minute levels of 3.3 mg % and greater, the correlation coefficient $r = 0.316$ indicates that no dependent relationship exists.

The effect of route of administration upon the awakening blood level was also investigated. Each of 6 rabbits received 35 mg/kg in the thigh muscle, 6 received 35 mg/kg intraperitoneally, 6 received 35 mg/kg subcutaneously in the flank, and 6 were given 50 mg per kilogram by stomach tube. In addition, 6 rabbits received 30 mg/kg in the marginal ear vein in order to determine the effect of a lower dosage level. The animals were placed on their backs when anesthetized and blood samples were taken as soon as they had righted themselves. The results are shown in Table I.

It will be seen that there was little variation between routes. The mean was found to be about 1.2 mg % for all routes with standard deviations of about 0.2 mg %. Evaluation of the differences between routes by means of the analysis of variance reveals the differences to be of no significance ($F = 0.38$).

2 Goldbaum, L., *J. Pharm. and Exp. Therap.*, 1948, **94**, 68.

*Summary.* 1. The correlation between the blood level of sodium pentobarbital and the duration of anesthesia in rabbits was investigated.

2. The awakening level was found to be quite constant at about 1.2 mg %, and was uninfluenced by route of administration or dose of the drug in the range tested.

3. No relation could be demonstrated between duration of anesthesia and the barbiturate blood level 10 minutes after injection, or with awakening blood level.

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## A Method of Preparing Collodion Particles for Serologic Agglutination.* (17344)

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The agglutination of collodion particles as a method of demonstrating union between antigen and antibody has been utilized by several investigators as a highly sensitive serologic technic.[1–3] However, difficulty in producing uniformly active pellets and the frequency of non-specific reactions have limited its general use. Cavelti[4] recently reviewed these points and suggested certain modifications of earlier methods to avoid their inherent difficulties. In the course of attempts made in this laboratory during the past two years to devise a serologic test for viral hepatitis, the use of the collodion particle agglutination technic was explored. A simple method of preparing collodion particles which give consistently reproducible results was devised. It is the object of this paper to describe this method.

*Preparation of collodion particles.* The general method of Loeb[5] modified by Cannon and Marshall[6] was used with certain alterations. All glassware used was sterile.

*Stock solution.* One and one-half pounds of collodion, U.S.P. Merck (non-flexible) is poured slowly into 2 liters of singly distilled water contained in a 4 liter glass beaker. The water is stirred constantly with a glass rod while the collodion is being added, and a mass of collodion separates. The water is decanted and the mass is then washed three times with distilled water, and is finally pressed by hand between several layers of bibulous paper to remove excess water. The mass of collodion is further dried in an incubator at a temperature of 37°C for 24 hours or until the odor of ether is no longer detectable. During the period of drying, the mass is broken up into smaller pieces to allow exposure of more surface. When dry, the mass of collodion is weighed, and a 5% solution in acetone is prepared in a water bath at a temperature of 37°C. Stirring with a glass rod expedites the solution. This stock solution is stored in the ice-box at 4° to 6°C.

*Suspension of pellets.* 150 ml of stock solution is poured into the glass container of a Waring Blendor. The base of the Blendor is wrapped with a damp towel, and another damp towel is draped over the open top of the glass container to avoid splashing. The apparatus is set before an open window in such a way that escaping fumes of acetone may be readily dispersed by currents of air. A glass funnel with the stem pulled to a fine capillary tip is placed on a ring stand so that the tip is approximately 2 cm below the top

* These investigations were conducted, in part, with the aid of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

1 Goodner, K., *Science*, 1941, **94**, 241.

2 Saslaw, S., and Campbell, C. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 559.

3 Lange, K., Gold, M. M. A., Weiner, D., and Simon, V., *J. Clin. Invest.*, 1949, **28**, 50.

4 Cavelti, P. A., *J. Immunol.*, 1947, **57**, 141.

5 Loeb, J., *J. Gen. Physiol.*, 1922, **5**, 109.

6 Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1940, **38**, 365.

of the container and over the vortex of the agitated fluid. The Blendor is set in motion, and through the funnel is added 60 ml of a mixture of three parts distilled water and one part acetone. When all the water-acetone mixture has been added, the Blendor is turned off, and a heavy gelatinous precipitate separates and settles to the bottom of the glass container. The faintly cloudy supernate is decanted into a filter flask containing 300 cc of cold, doubly distilled water, and the resultant mixture becomes cloudy. The gelatinous precipitate in the glass container is redissolved in 150 ml of stock solution. The Blendor is again set in motion, 60 ml of a 3:1 water-acetone mixture is added, and the supernate is subsequently decanted from the gelatinous precipitate into the original filter flask containing the 300 cc of doubly distilled cold water and the first supernate. The gelatinous precipitate remaining in the glass container is redissolved in 100 ml of acetone, agitated with the Blendor, and precipitated with proportionate amounts of a 3:1 mixture of water and acetone. This procedure is repeated 2 or 3 times and the resultant supernates are decanted into the original filter flask containing the 300 cc of cold, doubly distilled water and the previous supernates.

The filter flask is then attached to a vacuum pump at a pressure of 25 lb until only a faint odor of acetone remains. This may be expedited by placing the filter flask in a water bath at 56°C. The cloudy suspension in the filter flask is then passed through a thin cotton filter to remove coarse collodion particles. The filtrate is centrifuged in an angle head centrifuge at 4000 r.p.m. for 20 minutes. The faintly cloudy supernate is decanted and discarded. The precipitate is resuspended and washed 3 times in doubly distilled water by centrifuging at a speed of 4000 r.p.m. for 10 minutes. The supernate after the last two washings should be almost clear. The washed particles are resuspended in doubly distilled water and made into a stock suspension of a density standardized so that a 1:10 dilution equals No. 2 of the McFarland Turbidity Scale.[7] Stock suspensions of pellets are kept in the ice-box. They may be stored at this temperature (4° to 6°C.) for 7 to 14 days, after which they tend to lose their capacity to enhance a known serologic response.

The suitability of each lot of pellets for use is determined by testing their capacity to be agglutinated by the interaction of Pneumococcus Type I polysaccharide and its homologous hyperimmune horse serum. To chemically clean, sterile, tubes (100 x 10 mm) containing 0.5 ml of polysaccharide in dilutions of $10^{-4}$ to $10^{-9}$ are added 0.1 ml of pellets, 0.3 ml of sterile 0.85% saline and 0.1 ml of a 1:5 dilution of hyperimmune horse serum. The mixture is shaken and allowed to stand one hour at room temperature, after which the tubes are spun at 1500 r.p.m. for 5 minutes in a horizontal head centrifuge. The tubes are then flipped, and the amount of agglutination is determined against a bright light with a No. 5 Magni-Focuser glass.† A control test is made in the same dilutions with normal horse serum. Characteristically the precipitin test as usually performed with Pneumococcus Type I polysaccharide and its homologous hyperimmune horse serum is positive in dilutions of antigen of $10^{-6}$. The pellets must be agglutinated (at least 1+) in a dilution of antigen of $10^{-8}$ for them to be suitable for use as an enhancing mechanism in detecting a serologic response. Many lots of pellets are agglutinated in a dilution of antigen of $10^{-9}$. No agglutination occurs in the normal horse serum control tests.

*Summary.* A simple and consistently reproducible method of preparing collodion particles in a Waring Blendor is described. Pellets made in this way are uniformly active for 7 to 14 days and give no non-specific reactions in the serologic system tested. In contrast to previously described methods which prohibit the use of metal agitators, the metal blades in the Waring Blendor caused no apparent difficulties in the preparation of pellets. Actually, the violence of the agitation produced by the high rotary speed of the blades appeared to assist in the production of more uniformly active and smaller pellets.

[7] McFarland, J., *J.A.M.A.*, 1907, **49**, 1176.

† Edroy Products Company, New York, N. Y.

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# Determination of Serum Antitrypsin in the Study of Malignant Neoplasia. (17345)

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Before 1920 an antitryptic activity in the blood of various species had been observed, and was the subject of several theories and investigations. Following this there was a period of about 20 years during which the problem was almost entirely neglected. In recent years attention has again been focused on the serum antitrypsin phenomenon, and no less than 10 methods for its study have been reported in the literature. Several of these have been designed for use in the clinical laboratory. Clark, *et al.*,[1] Duthie, *et al.*,[2] Tallan, *et al.*,[3] and others have reported the application of their respective methods to the study of cancer. Although the results obtained by these investigators are in no sense conclusive, there is a prevailing suggestion that serum antitrypsin is a reflection of improtant physiologic mechanisms and that its alteration in disease should in some manner serve the practical objectives of clinical case study. At least 3 medical problems have been dealt with repeatedly in these reports: (1) endocrine problems, particularly pregnancy and its complications (2) infectious diseases, especially tuberculosis and syphilis, and (3) cancer.

Our interest in serum antitryptic activity persists upon the hope that certain changes may be of value in the diagnosis or study of malignant disease. The method devised in this laboratory[4] has the advantages of speed, simplicity and reproducibility, making it a suitable tool for extended clinical study. The survey reported herein will summarize our results to date.

*Materials and methods.* Our patients were unselected and included all those admitted to the M.D. Anderson Clinic during the interval of study. Whole blood samples of 3 cc each were drawn and transferred to centrifuge tubes containing 6 mg of crystalline lithium oxalate. The plasma was obtained and serial dilutions with physiological saline were prepared according to the method previously reported.[4] When a large number of plasma samples were to be run simultaneously we found that the procedure could be simplified by using only 4 dilutions (1:40, 1:80, 1:160, and 1:320). The linear segment of the sigmoid curve which expresses the tryosine liberation, and is important in determining the unitage of antitrypsin, almost invariably fell within this range of plasma concentration. Normal, control values were established by determination of the activity in the plasma of individuals who were clinically free of disease.

Of the 174 patients included in this series 81 had some type of malignant neoplasm. In 38 there were known metastases, and in 10 there was concomitant disease. After excluding 15 cases in which the diagnosis was not entirely clear, the remaining 159 were grouped into the several categories shown in Table I. More detailed analysis of the case records failed to reveal other correlations of importance.

*Results.* In the tabulation of our results we have accepted 140 units of antitryptic activity per cubic centimeter of plasma as the upper limit of normal. All our cases of acute infection, pregnancy and fibrocystic disease of the breast showed values in the pathological range. Patients with chronic infections, cardiovascular disease and diabetes showed antitryptic activity within the limits of normal. The malignant diseases were not sharply seg-

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1 Clark, D. G. C., Cliffton, E. E., and Newton, B. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 276.

2 Duthie, E. S., and Lorenz, L., *Biochem. J.*, 1949, **44**, 167.

3 Tallan, H. H., Cliffton, E. E., and Downie, G. R., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 667.

4 Wells, B. B., Marvin, H. N., and Waldvogel, M. J., *Am. J. Clin. Path.*, 1949, **19**, 448.

TABLE I.

| | Diagnosis | No. cases | Unitage Range | Unitage Avg |
|---|---|---|---|---|
| Antitrypsin <140 units | Non-malignant | | | |
| | No disease | 52 | 74-141 | 112 |
| | Cardiovascular disease | 9 | 69-137 | 104 |
| | Chronic inflammation | 11 | 76-162 | 129 |
| | Diabetes | 2 | 127-135 | 131 |
| | Malignant | | | |
| | Terminal malignancy | 3 | 117-123 | 121 |
| | Chronic myelogenous leukemia | 2 | 132-142 | 137 |
| | Primary skin cancer | 13 | 98-146 | 125 |
| | Primary cervical cancer | 5 | 93-142 | 120 |
| Antitrypsin >140 units | Non-malignant | | | |
| | Acute infections | 9 | 158-229 | 178 |
| | Fibrocystic disease of the breast | 2 | 162-166 | 164 |
| | Pregnancy | 3 | 166-219 | 196 |
| | Malignant | | | |
| | Primary skin and cervical cancer with acute infection | 5 | 162-193 | 178 |
| | Primary cancer other than skin and cervix | 13 | 138-204 | 157 |
| | Acute leukemia | 2 | 155-214 | 185 |
| | Metastatic cancer of all types | 38 | 151-240 | 182 |

regated on this basis, but there are interesting and well-defined trends. Individuals having extensive malignancies in terminal phase and those having small primary lesions showed no rise in plasma antitrypsin. In the first instance we may suppose that reactivity has been lost and, in the second, that the stimulous was insufficient. Visceral cancer and advanced, but not terminal, malignancy was quite regularly attended by high levels of plasma antitrypsin.

*Discussion.* The antitrypsin content of plasma is not altered in the early and more limited forms of malignant disease. It fails, therefore, to have positive diagnostic value when the condition is most amenable to surgical or radiological intervention. The determination also fails as a means of excluding cancer since terminal, and primary skin and cervical cancer are associated with values within the normal range.

Potentially, the greatest advantage of the procedure may be that the presence of hidden or metastatic cancer is suggested in those patients with more than 140 units of antitrypsin. It is true that in several non-malignant conditions the antitrypsin values are elevated above normal, but these are readily diagnosed by other means. In any event, the underlying mechanisms of the phenomenon present an interesting field for further investigations.

Both clinical and experimental studies indicate that the plasma antitrypsin concentration is subject to endocrine influence. High levels are regularly found during pregnancy. One is tempted to explain the findings in fibrocystic disease of the breast as a reflection of endocrine imbalance. In this connection we have recently studied a case of seminoma of the testis with pulmonary metastases in which plasma antitrypsin values varied between 219 and 204 units prior to x-ray therapy. During x-ray treatment the antitrypsin decreased rapidly to 147 units while the patient's general condition improved markedly. Values between 147 and 154 were obtained during a period of 6 weeks. At this time stilbestrol therapy was instituted. The plasma antitrypsin increased rapidly to 209 units in less than 3 weeks while the patient showed marked progress of his disease. Since estrogen administration has failed to increase plasma antitrypsin activity in animals we are inclined to attribute these results entirely to fluctuations in growth of the tumor. Plasma antitrypsin in relation to testicular neoplasms deserves considerably more study.

*Conclusions.* 1) The plasma antitrypsin test

does not show changes in association with early or minimal lesions of cancer.

2) The test may aid in excluding the diagnosis of advanced but non-terminal malignancy, or in the discovery of these lesions when they present themselves in obscure form

3) Since antitrypsin values appear to be closely related to the growth of seminoma of the testis, it suggested that the test may have unique prognostic value in the study of this condition.

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## Rat Growth Assay for Vitamin $B_{12}$. (17346)

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The method of assay for vitamin $B_{12}$ herein described was developed using a diet based on purified casein. The diet was made to contain 0.05% iodinated casein (Protamone) as a metabolic stimulant and 0.5% sulfaguanidine to inhibit bacterial synthesis of vitamins. Weanling rats, depleted on this diet for about 7 days, show a depression of growth rate which is counteracted by administration of vitamin $B_{12}$. The rate of response within a critical range is proportional to the amount of vitamin $B_{12}$ administered, either as the purified vitamin or as concentrates of the vitamin, such as liver extract.

It is well known that liver extract and vit. $B_{12}$ show much higher activity by injection than by oral administration in the pernicious anemia patient. We were interested in the present study to determine whether a difference could be shown between the effects of oral and intramuscular administration of the vitamin. We were interested also to determine the comparative effects of single doses at the beginning of the assay period versus divided doses throughout the assay period. Following completion of these studies, Emerson[1] reported that vitamin $B_{12}$ is equally active by either the oral or subcutaneous route in daily doses ranging from 0.0625 to 0.5 $\mu$g per rat day, somewhat higher than the range we have studied.

Register, Ruegamer and Elvehjem[2] reported a quantitative response in rat growth in their assay in the range of 0.025 to 0.1 U.S.P. units per rat day in the form of commercial liver extract. Direct comparison with vit. $B_{12}$ activity was not made in the Wisconsin report. We were interested to determine with a sample liver extract whether 1 $\mu$g of vit. $B_{12}$ would correspond fairly closely by rat assay to I U.S.P. unit, a figure previously suggested by Rickes *et al.*,[3] to be in approximately the correct range.

*Experimental.* Weanling rats, 35-45 g, of mixed sex were placed on a vitamin $B_{12}$ low diet for depletion. The $B_{12}$ depletion diet has the composition shown in Table I. In preliminary assays S.M.A vitamin test casein was further purified by the method of solution and reprecipitation of Novak and Hauge.[4] Later we found that vitamin test casein gave satisfactory results without further purification.

The rate of weight gain is about 12-15 g during the second week on the depletion diet and diminishes to about one-half this rate during the third week of depletion. The depletion period used ranged from 7 to 14 days. The 7-day period is preferred because of the increased mortality which occurs on more ex-

1 Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 392.

2 Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

3 Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

4 Novak, A. F., and Hauge, S. M., *J. Biol. Chem.*, 1947, **174**, 647.

TABLE I.
Vitamin $B_{12}$ Depletion Diet.

| | g/100 g |
|---|---|
| Purified casein | 18 |
| Cystine | 0.2 |
| Dextrin | 69 |
| Salt mixture No. 1 (U.S.P.) | 4 |
| Cellu-flour | 2 |
| Agar | 1.5 |
| Primex | 5 |
| CLO | 1 |
| Choline | 0.1 |
| Sulfaguanidine | 0.5 |
| Protamone (iodinated casein) | 0.05 |
| | mg/100 g |
| Riboflavin | 3 |
| Thiamine · HCl | 3 |
| Inositol | 20 |
| Niacin | 3 |
| Pyridoxine · HCl | 5 |
| Biotin | .01 |
| Folic acid | .01 |
| Calcium pantothenate | 5 |
| p-Amino benzoic acid | 5 |
| Menadione | 0.5 |

tended depletion. Groups of about 50 rats are depleted at one time with about 16 rats to a cage. Following depletion, the rats are placed in individual cages with raised floors. Six rats of mixed sex are used for each assay level. As a check on the rat assay, experiments were run, using the dietary regimen and procedure for assay of the animal protein factor (zoopherin), as described by Zucker and Zucker.[5] Following the preferred method described by these authors, we fed diet P60, based on 60% cottonseed meal to the mother rats during gestation and lactation, and to the offspring to 28 days of age when the curative assay was begun. Five male rats were used in each group.

*Oral vs. Injection Administration.* Three vit. $B_{12}$ preparations studied were as follows: a concentrate of vit. $B_{12}$, 25 $\mu$g per cc, supplied for investigational use by Merck and Company (designated Concentrate No. 1); a concentrate of vit. $B_{12}$ prepared in this laboratory (H.H.F.) to contain any desired concentration of vit. $B_{12}$ as determined by microbiological assay (designated Concentrate No. 2) and Cobione (Merck), 10 $\mu$g crystalline vit. $B_{12}$ per cc. Ampoules of Concentrate No. 1 were used as the standard throughout. A lot of these ampoules was assayed microbiologically by Eleanor Willerton in our laboratories against a lot of material distributed by Merck and Co. as a microbiological assay standard. By repeated comparisons, ampoules of Concentrate No. 1 were found to contain very close to 25 $\mu$g of vit. $B_{12}$ per cc.

Experiments were devised to compare the activity of vitamin $B_{12}$ added directly to the diet and given orally, or by injection, in single or multiple doses. For direct addition to the diet, solutions of vit. $B_{12}$ at a level of about 1 $\mu$g per cc were mixed with about 20 g of starch. The starch was then dried in air at 50-60°C and added to one kg of the basal diet. Results of assays wherein vit. $B_{12}$ was added directly to the diet are shown in Table II.

For comparison of oral versus injection dosage under conditions of multiple dosage, vit. $B_{12}$ solutions were made to contain from .33 to 1.0 $\mu$g per cc. Intramuscular injections and oral feedings of 0.23 cc per dose were then made 3 times weekly over the 2-week assay period at various assay levels. When comparing the effect of single oral or injection doses, appropriate critical concentrations of $B_{12}$ were administered the first day of assay corresponding to a range of .025 to .075 $\mu$g per rat day for the 14-day period. The results of the different modes of administration are shown in Table III.

Experiments were designed also to relate the activity of vitamin $B_{12}$ in a very preliminary way with the labeled A.P.A. potency of liver extract preparations of known clinical activity. The microbiological assay method of Skeggs, Huff, Wright and Bosshardt[6] provided a further basis for correlation. Aseptic addition of the liver extract supplements to the microbiological medium was used to avoid destruction of the vit. $B_{12}$ which, according to Stokstad *et al.*,[7] occurs on autoclaving.

The results of the comparison assays be-

[5] Zucker, L. M., and Zucker, T. F., *Arch. Biochem.*, 1948, **16**, 115.

[6] Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **176**, 1459.

[7] Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

TABLE II.
Direct Addition of Vitamin $B_{12}$ to the Diet.

| Exp. | Supplement | Vit. $B_{12}$* per kg diet, $\mu$g | Avg 2 wk wt gain and std. error,† g |
|---|---|---|---|
| 1 | None | 0 | 21‡ |
| | Cobione | 5 | 39 ± 3.5 |
| | ,, | 10 | 53 ± 3.7 |
| | ,, | 30 | 49.4 ± 4.2 |
| 2 | None | 0 | 17.6 ± 6.3 |
| | Vit. $B_{12}$ concentrate No. 1 | 5 | 25.3 ± 0.2 |
| | Vit. $B_{12}$ concentrate No. 1 | 15 | 39.3 ± 5.5 |
| 3 | None | 0 | 18.1 ± 3.1 |
| | Vit. $B_{12}$ concentrate No. 1 | 10 | 38.5 ± 3.9 |
| | Vit. $B_{12}$ concentrate No. 2 | 10 | 37.2 ± 4.3 |
| | Vit. $B_{12}$ concentrate No. 2a | 10 | 40.6 ± 2.2 |

* The preparations used were checked for vitamin $B_{12}$ content by microbiological assay, one against the other, and against a Merck microbiological assay standard. See text.

† Standard error = $\frac{\sqrt{\Sigma d^2}}{\sqrt{n(n-1)}}$.

‡ Only 3 of 6 rats survived.

TABLE III.
Vitamin $B_{12}$ Assays—Injection and Oral Administration.

| Exp. | Supplement | Avg 4 wk wt gain and range, g |
|---|---|---|
| Assay method of Zucker and Zucker | None | 35.4 ( 31- 41) |
| | Vit. $B_{12}$ conc. No. 1, 10 $\mu$g per kg diet | 123.2 (113-140) |
| | Vit. $B_{12}$ conc. No. 1, = .05 $\mu$g daily by mouth* | 110.1 ( 90-138) |
| | Vit. $B_{12}$ conc. No. 1, = .05 $\mu$g daily by inj.* | 123.6 (108-140) |
| | Vit. $B_{12}$ conc. No. 2, = .05 $\mu$g daily by inj.* | 115.0 (110-120) |
| | | Avg 2 wk wt gain and stand. error |
| Method in study | None | 18.2 ± 4.5 |
| | Vit. $B_{12}$ conc. No. 1, 0.35 $\mu$g inj. (single dose) | 26.0 ± 2.9 |
| | Vit. $B_{12}$ conc. No. 1, 0.35 $\mu$g oral (single dose) | 28.5 ± 5.5 |
| Method in study | None | 17.7 ± 3.7 |
| | Vit. $B_{12}$ conc. No. 1, = .033 $\mu$g daily by inj.* | 27.3 ± 9 |
| | Vit. $B_{12}$ conc. No. 1, = .067 $\mu$g daily by inj.* | 35 ± 3.7 |
| | Vit. $B_{12}$ conc. No. 2, = .1 $\mu$g daily by inj.* | 41.1 ± 3.3 |

* Injections and oral dosings—3 times weekly to supply daily equivalent shown.

tween vit. $B_{12}$ and 2 injectable liver extract preparations are shown in Table IV. The microbiological assay of Liver Extract No. 1 indicated a vit. $B_{12}$ content of one $\mu$g per U.S.P. unit This value appears to be confirmed by the rat assay. Liver Extract No. 2, which showed a minimal clinical response, was found to contain only .22 $\mu$g of vit. $B_{12}$ per estimated U.S.P. unit by microbiological assay. Good agreement with this low value was indicated by the rat assay response. A resumé of experience in this laboratory in the preparation of experimental liver extracts for clinical trial has indicated in general that preparations low in vit. $B_{12}$ are correspondingly low in clinical activity.

*Discussion.* An advantage of the method described herein is the ability to use animals weaned directly from mothers on stock diet, without special dietary control of the parent animals. This is true at least for animals reared in our own stock colony. Weanling rats

TABLE IV.
Vitamin $B_{12}$ Rat Assay—Liver Extracts.

| Exp. | Supplement | Avg wt gain and range 2 wk, g | 3 wk, g | 4 wk, g |
|---|---|---|---|---|
| Assay method of Zucker and Zucker | None | 44 | 54 | 74 (59- 84) |
| | Vit. $B_{12}$ conc. No. 1, 5 $\mu$g/kg diet | 63 | 80 | 100 (85-128) |
| | Liver extr. No. 1, 5 U.S.P. units/kg diet | 65 | 80 | 98 (78-116) |

| | | Avg 2 wk wt gain and stand. error, g |
|---|---|---|
| Method in study | None | 17.7 ± 2.7 |
| | Vit. $B_{12}$ conc. No. 1, = .05 $\mu$g daily (i.m.)* | 36.8 ± 4.4 |
| | Liver extr. No. 1, + .05 U.S.P. unit dally (i.m.)* | 34.6 ± 3.6 |
| Method in study | None | 17.7 ± 3.7 |
| | Vit. $B_{12}$ conc. No. 1, = .1 $\mu$g/day | 41.1 ± 3.3 |
| | Liver extr. No. 1 = 0.1 U.S.P. unit/day (i.m.)* (Equal to .1 $\mu$g $B_{12}$ by *L. leichmannii* assay) | 44.8 ± 5 |
| | Liver extr. No. 2 = 0.2 U.S.P. unit/day (i.m.)* (Equal to .044 $\mu$g $B_{12}$ by *L. leichmannii* assay) | 34.5 ± 4.2 |

* Injections made intramuscularly 3 times per week in doses to supply the daily equivalent shown.

from outside sources have not been tried.

The depletion period following weaning must remain, to some extent, a matter of judgment, depending on the condition of various lots of animals. It appears necessary to deplete the animals until the controls gain an average of only about 20 g during the 2-week period of assay. This has held true with a 7-14 day depletion in all but one of 12 assays in this laboratory involving about 600 rats.

No significant difference has been noted between the response of male and female rats in these studies, and precautions other than equal pairing as to sex do not appear necessary or desirable on the basis of convenience. Although there is considerable variation in response among individual animals, as shown by the fairly high standard errors (Tables II-IV), the significance of the results also appears high because of the distinct spread in increments between the control and test groups. In a few instances assays have been extended for 3 weeks. In these cases the animals supplemented with vit. $B_{12}$ continued to gain at a steady rate, whereas the control animals showed a small and constantly declining rate of weight gain, plus an increasing mortality. Occasional control rats gain weight rapidly without any supplement. We surmise that these unusual animals have a high rate of intestinal synthesis of vit. $B_{12}$, similar to the fast growing rats described by Hartman, Dryden and Cary,[8] and that neither the sulfaguanidine nor iodinated casein affect these particular rats nearly as much as they do the general run of test animals. In the above-mentioned single assay run in which the animals were not sufficiently depleted before assay, the control group gained weight nearly as rapidly the first 2 weeks as the supplemented groups. The groups were continued another week during which the supplemented groups gained considerably more weight than the controls, indicating that depletion of the control group had finally taken effect.

Our findings are in accord with those of Lillie, Denton and Bird[9] with chicks indicating that a single administration of vitamin $B_{12}$ at the beginning of assay meets the needs of deficient animals for growth for at least an ensuing 2-week period. Thus the assay may be simplified to involve only one administration of concentrated materials, such as liver extract, which lend themselves to this technic. An advantage of the single dosage technic is found in supplying the deficient

[8] Hartman, A. M., Dryden, L. P., and Cary, C. A., *Fed. Proc.*, 1949, **8**, 205.

[9] Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, **176**, 1477.

animals the full supplement at a time most conducive to overcoming the acute phase of the deficiency.

The present data indicate that vit. $B_{12}$ is about equally effective by oral or injection administration in promoting growth of young rats on highly specialized depletion diets. The deficiency of vit. $B_{12}$ induced in rats by these rather extreme dietary means does not, therefore, appear to evoke a failure in utilization of the vitamin, but rather a true deficiency and enhanced need for the vitamin itself.

The following additions to the diet failed to yield a comparable growth response, and even appeared to have an inhibitory effect: 5-10% purified casein, 5-10% of various primary-grown and brewer's yeasts, and additional folic acid. The addition of thymidine, 25-50 mg per kg diet, betaine hydrochloride 0.5%, or additional choline 0.1% likewise did not stimulate growth in absence of vit. $B_{12}$.

The growth promoting action of vit. $B_{12}$ in rations containing iodinated protein has recently been reviewed.[10] Betheil and Lardy[11] have further compared the effectiveness of vit. $B_{12}$, whole liver substance and extracts high in APA activity, as growth promoting materials for hyperthyroid rats. The ration used by these authors was also based on purified casein, but did not contain a sulfa drug. It is worthy of note that mortality is high among our control animals and that death is generally preceded by appearance of hemorrhage about the nose and paws. Vit. $B_{12}$ clearly prevents this syndrome and has a marked effect toward longevity under the dietary conditions imposed.

The question of the equivalence of vit. $B_{12}$ with U.S.P. anti-pernicious anemia units can only be answered by assays in human pernicious anemia patients. Microbiological and animal assays may serve as a useful guide, however, toward this goal. The data presented provide some evidence that concentrated liver extracts which show a correlation by microbiological assay of 1 U.S.P. unit approximately equal to 1 $\mu$g of vit. $B_{12}$, show a similar approximate equivalence by rat assay.

*Summary.* The growth response of vit. $B_{12}$ depleted rats under the conditions studied is proportional to the vit. $B_{12}$ administered in the critical range of .025-.1 $\mu$g per rat day. Oral and injection administration of the vitamin at critical levels yield approximately equal growth responses. Addition of the vitamin to the diet, multiple dosing or single dosing the first day of assay also appear roughly equivalent. A preliminary correlation between the results of microbiological and rat assays as applied to injectable liver extracts was obtained.

Received August 4, 1949. P.S.E.B.M., 1949, **72**.

[10] *Nutrition Reviews*, 1949, **7**, 183.

[11] Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, **37**, 495.

## Mucoproteins of Human Plasma. III. Electrophoretic Studies of Mucoproteins from Perchloric Acid Filtrates of Plasma.* (17347)

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It has been shown[1] that the "proteose" of human plasma is a mixture of mucoproteins with isoelectric points which are low in comparison with those of the principal plasma proteins. The present communication deals in more detail with the electrophoretic behav-

* The electrophoresis studies were supported by a grant from the United States Public Health Service and the isolation studies were supported by the American Cancer Society. Contribution No. 224 from the Department of Biochemistry and Nutrition, University of Southern California, School of Medicine.

[1] Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 609.

ior of this material.

*Methods. Preparation of materials.* The mucoprotein fractions were isolated in the manner already described from perchloric acid filtrates of pooled, normal, human plasma, and in one case (Preparation No. 44) from the plasma of a patient with gastric carcinoma whose plasma mucoprotein-tyrosine level was 4.9 mg % in comparison to normal levels of 2-4 mg %.[2] We are indebted to Dr. M. P. Petermann of the Memorial Hospital of New York City for the lyophilized sample of this plasma. Preparations were thoroughly dialyzed after isolation, and were lyophilized.

*Chemical characterization.* The lyophilized samples were analyzed for carbohydrate, glucosamine, nitrogen, and tyrosine† by the methods previously described.[1]

*Electrophoresis.* Samples were prepared for electrophoresis by dissolving them in the desired buffer, with subsequent dialysis against two portions of buffer for a period of at least 24 hours in each portion of buffer. The temperature was 5°C during the dialysis.

In most cases a small amount of insoluble material was present, and solutions were centrifuged immediately before electrophoresis. In order to cover a wide range of pH values with each preparation, it was necessary to recover material from the electrophoresis cell and use it in subsequent measurements. Recovered solutions were concentrated by placing them in a dialysis bag and blowing air over the bag at room temperature. This procedure, and the limited amounts of material available, made it impractical to control the concentrations, so that there was considerable variation in protein concentration in different experiments. It also seems probable that some change in composition took place during repeated recovery and concentration. This has contributed to the difficulty of identifying components at different pH values in some instances.

The buffer systems were prepared with diethyl barbiturate, acetate, citrate or phosphate. All buffers were made to give a final concentration of 0.08 M NaCl and 0.02 M with respect to the monosodium salt of the buffer system or the disodium salt in the case of phosphate. HCl was added to obtain the desired pH, so that the ionic strength was 0.1 in each case except at pH 6, where disodium phosphate was employed In this case the ionic strength was correspondingly greater.

Electrophoresis was carried out in an 11 ml Tiselius cell at 2.0°C, with a potential gradient of between 5 and 6 volts per cm. The conductivities used to calculate the potential gradient were also determined at 2°C on the buffer used to fill the electrophoresis cell. The pH of the buffer, also determined after dialysis, was obtained with a Beckman pH meter at room temperature.

The boundaries were photographed, generally after 3 hours, using the Philpot optical system,[3] and at a magnification of 1.05 to 1. The photographs were enlarged somewhat over 2 times for making the required measurements.

*Experimental results.* The preparations used in this study had the chemical characteristics shown in Table I. These preparations show the characteristically high carbohydrate and glucosamine content previously reported.

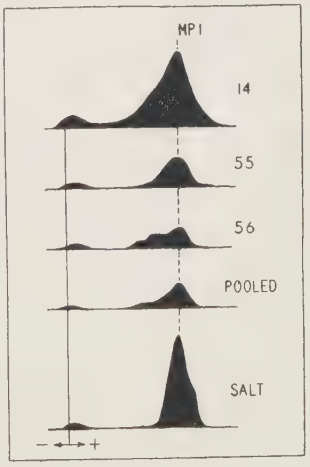


FIG. 1.

Electrophoresis patterns of mucoprotein preparations at pH 8.4. Descending boundaries. Preparations No. 14, No. 55, No. 56, and "pooled," are from perchloric acid filtrates of pooled, normal plasma. The "Salt" preparation was prepared entirely by ammonium sulfate fractionation, and contains 31% albumin.

2 Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 617.

† Although reported as tyrosine, it is recognized that the Folin's phenol reagent used is not specific for this amino acid.

3 Philpot, J. S. L., *Nature*, 1938, **141**, 283.

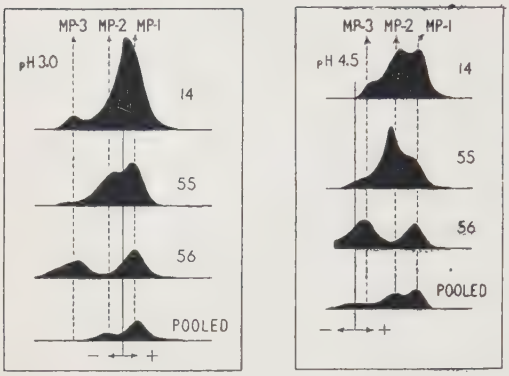


FIG. 2.
Electrophoresis patterns of mucoprotein preparations at pH 3 and 4.5. Boundaries descending toward the anode.

The electrophoresis patterns of the descending boundaries obtained on some of the preparations from human plasma are reproduced in Fig. 1 and 2. In the patterns of Fig. 1, obtained at pH 8.4, is included the pattern of a preparation made entirely by ammonium sulfate fractionation without preliminary deproteinization with perchloric acid. Although this preparation contains 31% albumin, it does demonstrate the greater degree of homogeneity which can be obtained with respect to the major mucoprotein component (MP-1) by such a fractionation procedure.‡ In Fig. 2, obtained at pH 3.0 and 4.5, are indicated components which we have designated as MP-1, MP-2, and MP-3. At pH 6 and higher, the resolution into well-defined components is less satisfactory and we have only indicated the position of MP-1 in Fig. 1.

In Fig. 3 we have attempted to present the pH-mobility curves for the descending boundaries of these components. Although we feel reasonably well satisfied with the general characteristics in the region below a pH of about 5.5, the uncertainty regarding MP-2 and MP-3 at higher pH's has been indicated in the figure.

*Discussion.* Human plasma mucoprotein prepared by the preliminary removal of other plasma proteins with perchloric acid may contain at least 3 electrophoretically different components, and the spreading of the boundaries suggests that the degree of heterogeneity is even greater than this. The proportions of the components vary considerably from preparation to preparation, and despite the rather drastic initial treatment with perchloric acid the preparations may be somewhat modified when solutions are repeatedly recovered by evaporation at room temperature. Because of these limitations, conclusions must be drawn with some caution. The preparations labeled No. 14, No. 55, and "pooled" are the most typical of preparations which we have presented for discussion. The analytical values which are given in Table I are in reasonably good agreement for these three. Preparation No. 56 had the least typical electrophoretic behavior and had the lowest values for glucosamine, carbohydrate, and tyrosine, except for the material prepared exclusively by salt fractionation, which contained 31 per cent albumin.

The one component which is seen in all preparations, including the "salt" preparation is MP-1. This material has a mobility of $-6.4 \times 10^{-5}$ cm$^2$ sec$^{-1}$ volt$^{-1}$ in the descending boundary at pH 8.4, and would appear in the $\alpha_1$-globulin fraction of serum at this pH. This component is still strongly negatively charged at pH 4.5, and is isoelectric at a pH of

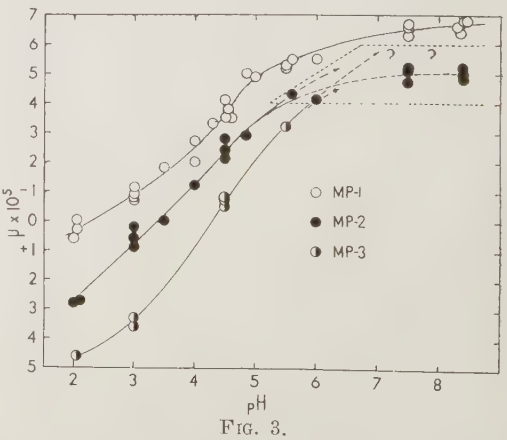


FIG. 3.
Mobility of mucoprotein components as a function of pH. Identification of components other than MP-1 is uncertain above pH 6, and this area of uncertainty is indicated by enclosing it in the dotted lines. Principally because of the spread of peaks, mobilities at all pH values are subject to considerable error.

‡ This preparation was made by Mr. Henry Weimer, and results of further refinements of this method will be reported in the near future.

TABLE I.

| Preparation | Source | Yield mg/100 ml plasma | Nitrogen, % | Glucosamine, % | Carbohydrate, % | Tyrosine, % |
|---|---|---|---|---|---|---|
| No. 14 | Human, normal | 38 | 8.1 | 10.1 | 15.6 | 3.9 |
| No. 44 | " cancer | 100 | 7.5 | 8.9 | 16.3 | 3.9 |
| No. 55 | Human, normal | 28 | 9.0 | 9.1 | 15.7 | 4.3 |
| No. 56 | " " | 32 | 7.1 | 8.1 | 13.6 | 3.4 |
| Pooled* | " " | — | 8.3 | 9.8 | 14.3 | 3.6 |
| Salt fract.† | " " | 72 | 7.25 | 7.6 | 13.2 | 3.1 |

* Five separately isolated lots were pooled for this sample.

† Contained 31% albumin by electrophoresis, assuming equal refractive increments per g of albumin and MP-1.

about 2.3 in citrate buffer.

At pH 8.4, some material slower than MP-1 is always seen, and at pH 4.5 there is generally a considerable amount of material with a mobility of about $-2.3 \times 10^{-5}$. This material which we have designated MP-2, has an isoelectric point at about pH 3.4. From the analytical data, it must also be a mucoprotein.

The material designated as MP-3 might conceivably be contaminated with albumin. The mobility is not very different from that of albumin in this buffer, and the broad peaks for MP-3 would generally overlap the mobility for albumin at pH 4.5. Small amounts of albumin might also contribute to the broadening of the electrophoretic peaks at pH 8.4. However, preparation No. 56 could not contain enough albumin to account for the peak observed at pH 4.5. Pending the isolation of more homogeneous preparations, the status of this component must remain in considerable doubt. The isoelectric point, however, is in the region of pH 4.3.

It is clear from the study of these preparations that mucoprotein components MP-1 and MP-2 should be demonstrable by electrophoresis of plasma at a suitable, more acid pH than is generally employed. Such a study has been made by Petermann *et al.*,[4] and further studies in this direction are being presented in paper IV of this series.[5]

The sample isolated from a patient with cancer (No. 44) presented essentially the same characteristics on electrophoresis as did the pooled sample. The similarity with respect to proportion of components was greater than the analytical data would have led one to suspect. This case, at least, would indicate that the increased amount of mucoprotein in cancer represents an increase in normal components rather than the appearance of an abnormal component.

Although the groups responsible for the low isoelectric points of these mucoproteins have not been identified, it seems likely that they are at least in part sulfuric acid ester groups. Sulfur in excess of that accounted for by cystine and methionine has been observed in preparations of mucoprotein from rat blood,[6] horse serum,[7] and human plasma.[1]

*Summary.* The electrophoretic behavior of mucoproteins isolated from perchloric acid filtrates of human plasma has been studied over the pH range of 2 to 8.4. There are at least 3 electrophoretic components in such preparations, the isoelectric points being approximately 2.3, 3.4 and 4.3. The major component has the lowest isoelectric point and travels with a mobility characteristic of $\alpha_1$-globulin at pH 8.4. The increase in the mucoprotein level isolated from the plasma of a patient with gastric cancer was largely in this acid mucoprotein fraction.

4 Petermann, M. P., Karnovsky, D. A., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

5 Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 110.

6 Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.*, 1944, **4**, 417.

7 Mayer, K., *Z. f. Physiol. Chem.*, 1942, **275**, 16.

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# Mucoproteins of Human Plasma. IV. Electrophoretic Demonstration of Mucoproteins in Serum at pH 4.5.* (17348)

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Mucoproteins in human plasma have been isolated and characterized chemically,[1] their increase in cancer has been discussed,[2] and the material isolated from plasma has been studied electrophoretically.[3] It was shown that in the usual electrophoresis pattern at pH 8.4 much of the mucoprotein should migrate with the $\alpha_1$-globulin, and would thus be included with any other material which contributes to the $\alpha_1$-globulin fraction. It was found, however, that the isoelectric points of the fractions contributing to the isolated mucoprotein were quite low, and that they would all remain negatively charged at pH 4.5. Such a pH would, then, appear more suitable for the electrophoretic demonstration of these mucoproteins in serum. It has been shown by Petermann *et al.*[4] that there is an acid component in human serum which may be demonstrated by electrophoresis at pH 4.0, and which increases in amount in those conditions in which the mucoprotein has been shown to be increased. They have suggested that this electrophoretic component may be the same mucoprotein as that with which we have been concerned, and the present studies were intended primarily to determine the extent to which such an identification can be made.

*Methods.* The methods employed in the electrophoresis studies were the same as those previously described.[3] All electrophoresis measurements were made at 2°C at an ionic strength of 0.1. At pH 8.4, the buffer was diethyl barbiturate, and at pH 4.5 the buffer was acetate. In both cases, the ion and undissociated acid of the buffer had a total concentration of 0.02 M. The pH is that determined at room temperature. Serum samples were generally diluted to 6 times their original volume, and thus had a total protein concentration in the neighborhood of one per cent. The patterns were photographed after 3 hours.

At pH 4.5, some precipitate forms during dialysis, and is removed before electrophoresis. The nature of this precipitate and its effect upon the electrophoresis pattern has not been thoroughly investigated, but the amounts of protein involved are small. Where protein concentrations are reported subsequently, they have been calculated from the total protein concentration of the serum, and the relative

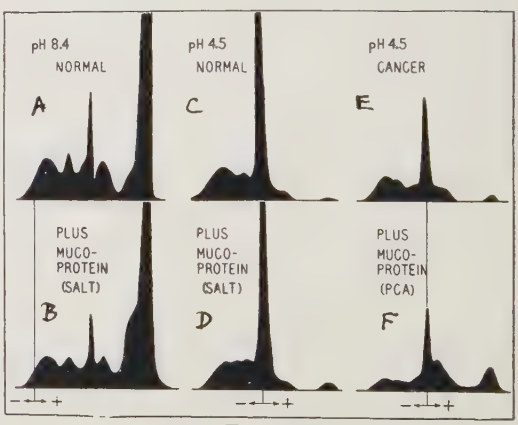


FIG. 1.

Electrophoresis patterns at pH 8.4 and pH 4.5. Boundaries descending to anode at pH 8.4, descending to cathode at pH 4.5.

A. Normal plasma at pH 8.4. B. Same normal plasma at pH 8.4 with mucoprotein (MP-1) added. C. Same normal plasma at pH 4.5. D. Same normal plasma at pH 4.5 with mucoprotein (MP-1) added. E. Serum from cancer patient at pH 4.5. F. Same cancer serum at pH 4.5, with mucoprotein added (MP-1, MP-2, and MP-3).

* This study was supported by a grant from the U. S. Public Health Service. Contribution No. 225 from the Department of Biochemistry and Nutrition, University of Southern California School of Medicine.

[1] Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 609.

[2] Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 617.

[3] Mehl, J. W., Humphrey, J., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 106.

[4] Petermann, M. P., Karnovsky, D. A., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

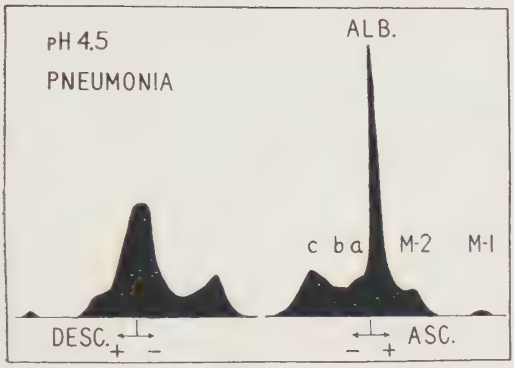


FIG. 2.

Electrophoresis patterns, ascending and descending, at pH 4.5, of serum from a patient with pneumonia. The designation of the components is shown.

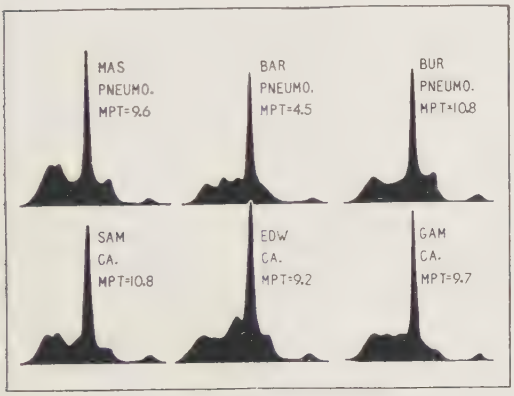


FIG. 3.

Electrophoresis patterns, descending to the cathode, at pH 4.5. The 3 upper patterns are from patients with pneumonia, the 3 lower patterns from patients with cancer. The value for MPT is that for the mucoprotein-tyrosine in mg%.

areas of boundaries in the electrophoresis pattern.

*Experimental results. Electrophoresis at pH 8.4.* In order to show that the mucoprotein does contribute to the $\alpha_1$-globulin fraction, a sample of normal plasma was subjected to electrophoresis with and without added mucoprotein. The mucoprotein fraction used for this study was one isolated by ammonium sulfate fractionation and consisted only of the fraction designated as MP-1,[3] contaminated with some albumin. The electrophoresis patterns obtained in this experiment are shown in Fig. 1-A and 1-B, and it is apparent that the mucoprotein MP-1 does indeed increase the $\alpha_1$-globulin fraction at pH 8.4.

*Electrophoresis at pH 4.5.* On the basis of the results obtained with the isolated mucoprotein, a pH of 4.5 was selected as being suitable for the demonstration of the mucoprotein in serum. In Fig. 2 are reproduced the ascending and descending boundaries obtained in the electrophoresis of serum from a patient (HUM) with pneumonia, indicating the division into components which we have employed, and their designations. We have designated the 2 acid components as M-1 and M-2 and the globulins as a, b, and c. The separation of the peaks is generally better in the boundary in which the globulins are descending and mucoprotein is ascending and only this boundary is given in the electrophoresis patterns of Fig. 1 and 3. Although the example in Fig. 2 is not the most satisfactory for illustrating the presence of components a, b, and c, an inspection of the patterns in Fig. 3 will indicate that such a division is justified. The acid components M-1 and M-2 are clearly evident in Fig. 2. The amounts of M-1 and M-2 are considerably elevated over the normal levels (compare Fig. 1-C). This has been true of all cases studied in which the chemical determination showed an elevation of the mucoprotein.

The electrophoresis pattern of the same normal serum which was used at pH 8.4 is shown at pH 4.5 in Fig. 1-C. As with the pathological serum of Fig. 2, there are two components corresponding to M-1 and M-2 but in considerably smaller quantities. In Fig. 1-D is shown the electrophoresis pattern at pH 4.5 of this same normal serum with the addition of the same mucoprotein preparation employed at pH 8.4. M-1 is increased, and M-2 is unchanged, suggesting that the MP-1 fraction of isolated mucoprotein may be identified with the M-1 fraction in serum. The mobility of $-4.2 \times 10^{-5}$ (in the ascending mucoprotein boundary) is in good agreement with that observed in the isolated mucoprotein.

In Fig. 1-E the electrophoresis pattern of serum from a patient with cancer is presented, and in Fig. 1-F the same serum with mucoprotein added. In this case, the mucoprotein was isolated from a perchloric acid filtrate of plasma by the method previously de-

TABLE I.

Analytical Values Obtained on Perchloric Acid Filtrates from the Sera of 3 Patients, GAM, BUR, and HUM. Concentrations are expressed as mg per 100 cc of original serum, and were obtained by the methods previously described.[1] Average values of the ratios of carbohydrate: tyrosine (CHO/T) and carbohydrate:glucosamine (CHO/G) obtained on mucoprotein isolated from normal human plasma[1] are given for comparison.

| Sample | Tyrosine, mg% | CHO, mg% | CHO/T | N, mg% | Protein (Biuret) mg% | Glucosamine, mg% | CHO/G |
|---|---|---|---|---|---|---|---|
| GAM carcinoma | 9.7 | 52.0 | 5.3 | 23.5 | 240.0 | 33.5 | 1.55 |
| BUR (pneumonia) | 10.8 | 55.0 | 5.1 | 27.5 | 270.0 | 33.5 | 1.64 |
| HUM (pneumonia) | 5.7 | 27.5 | 4.8 | 14.5 | — | 21.0 | 1.31 |
| Normal | — | — | 3.6 | — | — | — | 1.27 |

scribed[1] and contained in addition to MP-1 the slower components (MP-2 and MP-3) seen in these preparations.[3] The addition of such material to serum is seen to result in an increase in both the M-1 and M-2 fractions of the resulting mixture. The mobility of the M-1 fraction in serum corresponds closely with that of the MP-1 component of isolated mucoprotein. The mobility of the M-2 fraction of serum, however, ($-1.4$ to $-2.0 \times 10^{-5}$) does not agree with the mobility of either the MP-2 ($-2.4$ to $-2.8 \times 10^{-5}$) or MP-3 ($-0.5$ to $-1.2 \times 10^{-5}$) of the isolated preparations. When such preparations are added to serum, we have been able to find satisfactory correspondence only between the mobilities of the M-1 and MP-1 fractions.

*The relation of the electrophoretic components M-1 and M-2 to chemically determined mucoprotein.* Electrophoresis patterns of a number of pathological sera at pH 4.5 are shown in Fig. 3. It will be seen that an increase in M-2 is found to accompany an increase in M-1 in either carcinoma or pneumonia. In each instance, the level of mucoprotein-tyrosine (MPT) in mg percent in the original plasma[2] is also indicated. The mucoprotein-tyrosine is between 9 and 11 mg percent in all cases except that of BAR, where it is 4.5. Inspection of the electrophoresis patterns will make it evident that the area of the M-1 peak is about the same in all except that of BAR, and that there is at least a rough correspondence between the amount of M-1 and the MPT. The amount of M-2, however, does not seem to bear any quantitative relationship to the amount of MPT.

Serum samples from three patients were studied in somewhat greater detail. Sufficient serum was obtained in each case to carry out the electrophoresis at pH 8.4 and pH 4.5; to prepare perchloric acid filtrates for the determination of the mucoprotein-tyrosine, as well as the carbohydrate, nitrogen, glucosamine, and the value of the biuret reaction.[1] The results of the chemical analyses of the filtrates are given in Table I, together with the ratios of carbohydrate:tyrosine and of carbohydrate:glucosamine for these filtrates and for the mucoprotein isolated from normal plasma. In Table II the amount of each electrophoretic component is calculated and compared with the amount of mucoprotein indicated by the chemical determination. This calculation is based upon a nitrogen content of 7.9%, determined upon the isolated mucoprotein. It must be recognized, of course, that the accuracy with which the areas of the small electrophoretic peaks for M-1 can be determined is not great, and that the translation of these areas into concentrations involves the assumption that the mucoprotein would have the same refractive index increment as that of the other serum proteins. It seems reasonable to conclude, however, that the agreement between the electrophoretic and chemical values indicates that the chemical determination is primarily a measure of M-1. Certainly, the sum of M-1 and M-2 is too great to be accounted for by the material in the perchloric acid filtrates.

In comparing the results of the electrophoresis experiments at pH 8.4 and 4.5, it is seen that M-1 does not exceed $\alpha_1$-globulin in any of these cases, but may be considerably smaller (as in the case of HUM). On the other hand, the amount of M-1 plus M-2 may be considerably larger than the amount of $\alpha_1$-globulin.

TABLE II.

Electrophoretic Analysis of the Sera of 3 Patients, GAM, BUR, and HUM. The concentrations are calculated from the total serum protein, assuming that the refractive index increment is the same for all components. The mucoprotein nitrogen represents the difference between that in perchloric acid and tungstic acid filtrates.*

| pH | Component | Concentration g/100 cc | | |
|---|---|---|---|---|
| | | GAM | HUM | BUR |
| 8.4 | Albumin | 3.3 | 2.9 | 2.2 |
| | $\alpha_1$–globulin | 0.3 | 0.6 | 0.4 |
| | $\alpha_x$– ,, * | — | — | 0.8 |
| | $\alpha_2$– ,, | 1.0 | 1.0 | 1.1 |
| | $\beta$– ,, | 0.6 | 0.6 | 0.7 |
| | $\gamma$– ,, | 0.9 | 1.2 | 1.3 |
| 4.5 | M–1 | 0.3 | 0.1 | 0.2 |
| | M–2 | 0.4 | 0.5 | 0.8 |
| | Albumin | 2.9 | 3.4 | 3.0 |
| | a | 0.7 | 0.5 | 0.6 |
| | b | 0.9 | 0.5 | 0.6 |
| | c | 1.1 | 1.2 | 1.1 |
| Mucoprotein —N × 12.5 | | 0.29 | 0.18 | 0.34 |

* The material designated $\alpha_x$–globulin is an abnormal component migrating between the $\alpha_1$– and $\alpha_2$–globulin.

*Electrophoretic Isolation of M-1.* Since the M-1 component becomes well separated from other components of serum at pH 4.5, it is well suited to electrophoretic separation. An experiment was carried out in which serum was undiluted, except for the dilution taking place during dialysis, and in which electrophoresis was carried out in an 11 ml analytical cell with 2 center sections. Electrophoresis was carried out, with compensation, until the ascending boundary of the M-1 component had nearly reached the top of the cell, and the ascending M-2 boundary was below the middle junction of the cells. One half of one channel of the cell could then be isolated, containing M-1 alone. This material was subjected to chemical analysis, with the results given in Table III. There can be no doubt that the material represented by the M-1 peak at pH 4.5 has the same general chemical characteristics as the mucoprotein isolated from perchloric acid filtrates of serum or plasma.

TABLE III.

Analytical Values for Electrophoretically Isolated M–1.

| | |
|---|---|
| Tyrosine | 7.6 mg%* |
| Carbohydrate | 34.4 ,, |
| CHO/T | 4.5 |
| Nitrogen | 16.2 mg% |
| Glucosamine | 28.5 ,, |
| CHO/G | 1.2 |

* These refer to concentration in the solution removed from the cell.

*Discussion.* The identity of the M-1 component of the electrophoresis pattern of serum at pH 4.5 and the fraction of the isolated mucoprotein which we have designated as MP-1 seems to be adequately established by the experiments presented. The addition of the isolated material to serum has confirmed the identity of the electrophoretic mobilities, and the material isolated by electrophoresis has been shown to have chemical properties similar to the material isolated by fractional precipitation.

The nature of the M-2 component of the electrophoresis pattern of serum at pH 4.5 is not established. The mobility does not correspond entirely with that of any component of the isolated mucoprotein. The increase in the M-2 component resulting from the addition of isolated mucoprotein containing fractions other than MP-1 would suggest that M-2 is also a mucoprotein. The association of increases in M-1 and M-2, and the rather low isoelectric point of M-2 would point in the same direction, but certainly without being conclusive. The solution of the problem will require the isolation of a homogeneous preparation which has the electrophoretic properties of M-2 by itself or on addition to serum. The isolation of such material is now being investigated.

*Summary.* When serum is subjected to electrophoresis at pH 4.5, two components are seen which have isoelectric points more acid than those of albumin. One of these has been identified with a mucoprotein which has been shown to increase in amount in cancer, pneumonia, and certain other diseases. The second component, with a less acid isoelectric point, may also be a mucoprotein, but this point has not been definitely established.

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## Effect of Adrenergic Blocking Agents on the Cutaneous Action of Epinephrine. (17349)

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Adrenergic blocking drugs are of clinical interest in the treatment of conditions characterized by the element of vasospasm, such as early Buerger's disease, Raynaud's disease and causalgic states. It was felt that by the direct diffusion or by ion transfer through the skin, it would be possible to avoid the generalized toxic manifestations encountered by way of the parenteral route.[1] The adrenergic blocking agents selected for this study were N-(2-chloroethyl) dibenzylamine hydrochloride (Dibenamine) introduced by Nickerson and Goodman[2] and N-(2-bromoethyl)-N-l-naphthalenemethylamine hydrobromide (SY-28) introduced by Loew and Micetich.[3] Prior to application in the human, data were obtained in a group of albino rabbits.

*Methods.* Albino rabbits weighing 2.0 to 4.5 kg were used. The abdomen and thorax of the animals were depilated with a special sulfide-detergent mixture[4] 24 hours prior to use. Solutions of Dibenamine and SY-28 in propylene glycol were freshly prepared prior to each experiment. Four-tenths ml of these solutions or of solvent controls were applied to rectangular strips of asbestos paper 1.25 by 12.5 cm in size. This amount of solution was sufficient to saturate the pads. The strips were then applied to the abdomen in 4 parallel sites in the following order: solvent without current, solution containing the drug without current, solution containing the drug plus current, solvent plus current. Two to four rabbits were used at each of the various concentrations of blocking agents studied. Since the active portions of both Dibenamine and SY-28 are cationic, the anode of a suitable direct current source* was used to transfer the agent into the skin. A current density of 0.33 ma/cm$^2$ or a total current of 5.2 ma was employed. This dosage of current was arbitrarily selected as being just below the pain threshold for human skin and was well tolerated by the rabbits. The solutions were placed in contact with the skin for exactly 5 minutes with or without current. Immediately following the 5 minute application, the asbestos pads were removed and the blocking agent wiped off with propylene glycol.

To measure the adrenergic blocking activity of the treated sites, 0.1 ml of 1:1,000, 1:10,000 and 1:40,000 epinephrine solutions were injected intracutaneously 30 minutes following application of the blocking agents. An equal amount of physiological saline served as a control. The injection sites were randomized. Vasoconstriction reached a max-

[1] Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, **3**, 3.

[2] Nickerson, M., and Goodman, L. S., *Proc. Am. Fed. Clin. Research*, 1945, **2**, 109.

[3] Loew, E. R., and Micetich, A., *Fed. Proc.*, 1947, **6**, 304.

[4] Pitesky, I., and Last, J. H., *Science*, 1948, **108**, 657.

* A McIntosh Wall Plate was used. This machine is manufactured by the McIntosh Electrical Corp., Chicago, Ill.

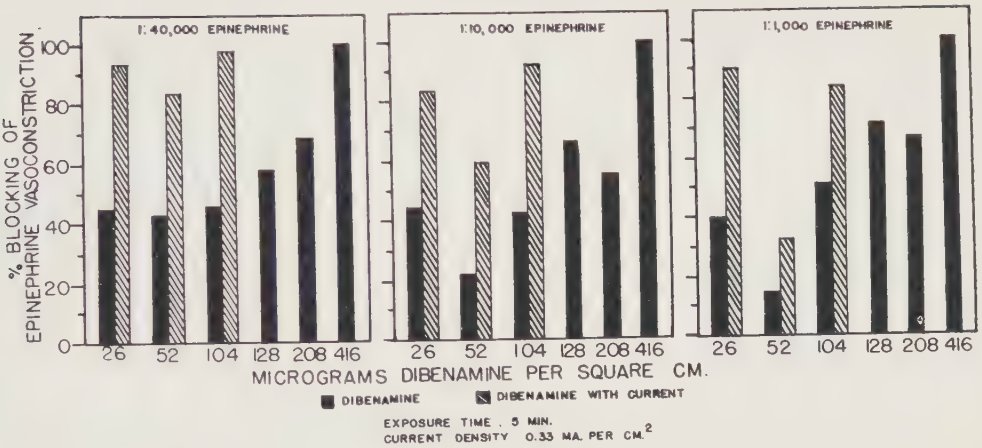


FIG. 1.

Effects of ion transfer on the cutaneous adrenergic blocking activity of Dibenamine.

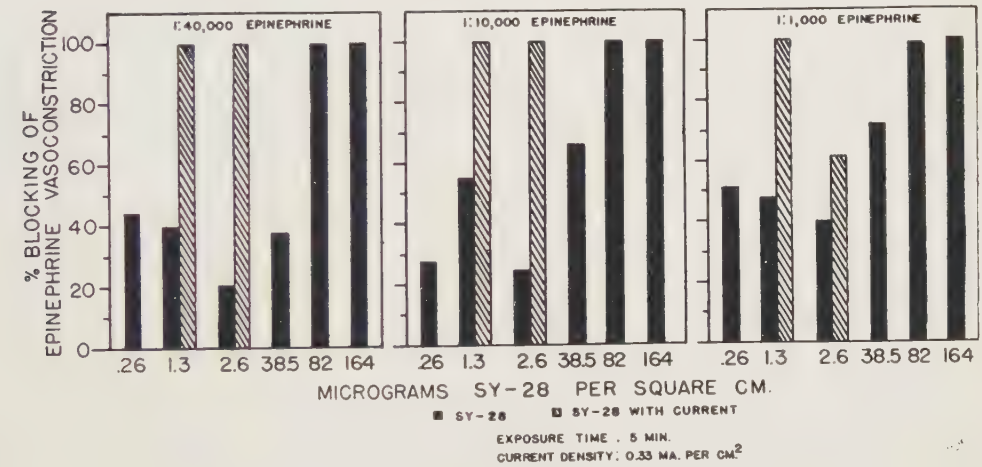


FIG. 2.

Effects of ion transfer on the cutaneous adrenergic blocking activity of SY-28.

imum in 30 minutes and manifested itself by a circular area of blanching.

The diameters of the blanched skin were measured and the areas computed. These areas were then weighted for intensity of vasoconstriction. This was accomplished by arbitrarily recognizing four degrees of response and assigning an intensity number for each degree. Full, faint, very faint, and no vasoconstriction, (*i.e.* complete blocking), were assigned the numbers 3, 2, 1 and 0 respectively. The area multiplied by the intensity factor yielded a weighted area of vasoconstriction. The percentage of blocking was then easily obtained mathematically by comparing the treated sites with the control sites. The results were analyzed statistically by the method of paired comparisons. After calculating the statistic "t", the probability value "P" was obtained from Fisher's "t" table.

*Results.* The data obtained for Dibenamine and SY128 are illustrated in Fig. 1 and 2. Since the absolute amount of drug present in the skin following exposure was unknown, the values plotted along the abscissae were the amounts actually applied to the skin.

Dibenamine produced complete blocking of epinephrine at a dose of 416 $\mu$g per sq cm whereas SY-28 produced the same effect with 82 $\mu$g per sq cm. Under these conditions SY-28 was approximately 5 times as potent as Dibenamine in blocking epinephrine. The ap-

plication of current enhanced the penetration of both drugs in a statistically significant manner. Analysis of the epinephrine responses in 4 rabbits following the use of Dibenamine in a concentration of 104 $\mu g/cm^2$ with and without ion transfer yielded the following "P" values at various epinephrine concentrations:

a. 1:1,000 - P = 0.01
b. 1:10,000 - P = 0.06
c. 1:40,000 - P = 0.04

Correspondingly, with SY-28 in 4 rabbits with and without ion transfer at a dosage of 1.3 $\mu g/cm^2$ the following P values were calculated for the concentrations of epinephrine used:

a. 1:1,000 - P = 0.04
b. 1:10,000 - P = 0.07
c. 1:40,000 - P = <0.01

Dibenamine and SY-28 were found to be fixed locally, since the skin surrounding the treated site responded to injected epinephrine in a normal manner. During the course of the study, it was noticed that an area of erythema developed in the sites injected with epinephrine prior to the appearance of vasoconstriction. This response was even more marked and persistent when vasoconstriction was completely blocked. The process of ion transfer produced minimal erythema which made it difficult to evaluate the subsequent erythematous response to epinephrine. For this reason two groups of 8 rabbits each were given Dibenamine or SY-28 by the intravenous route. Epinephrine was injected intracutaneously as in the previous experiments 30 minutes after the injection of the blocking drug. Erythema developed rapidly and either disappeared over a 15 minute period or was followed by vasoconstriction in the situations where incomplete blocking occurred. This "epinephrine reversal" type of phenomenon suggests the presence of vasodilator fibers in the skin of the rabbit.

Fig. 3 and 4 illustrate a dose response relationship when the blocking agents were given intravenously. One hundred percent blocking of epinephrine vasoconstriction occurred when 32 mg of Dibenamine per kilo were given. The same effect was achieved with SY-28 when 4 mg per kilo were injected. Thus

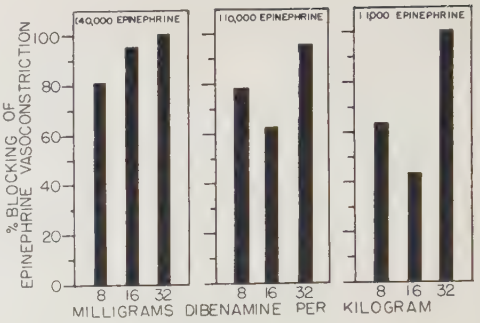


FIG. 3.
Cutaneous adrenergic blocking activity of intravenous Dibenamine.

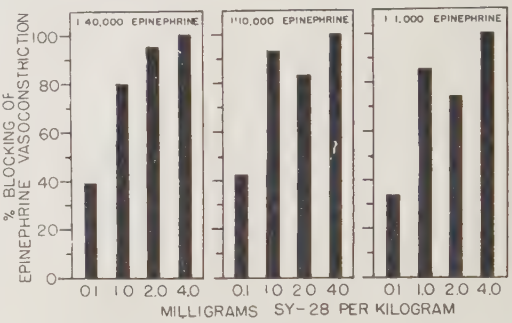


FIG. 4.
Cutaneous adrenergic blocking activity of intravenous SY-28.

SY-28 was approximately 8 times as potent as Dibenamine when given by the intravenous route.

*Summary.* 1) Both Dibenamine and SY-28 were absorbed by the intact rabbit skin and were capable of blocking the vasoconstriction produced by intracutaneously administered epinephrine. By this route SY-28 was approximately 5 times as potent as Dibenamine.

2) When administered by ion transfer, the penetration of both drugs into the skin was significantly facilitated.

3) Both blocking compounds were fixed locally in the skin. Contiguous untreated areas were still capable of responding to epinephrine.

4) By the intravenous route, SY-28 was approximately 8 times as potent as Dibenamine.

5) The "epinephrine reversal" phenomenon has been noted for the first time in the skin of the rabbit and suggests the presence of vasodilator fibers.

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# Stripping Film Technic for Radioautographs. (17350)

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(Introduced by E. M. Landis.)

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The method of radioautography for localization of radioactive tracers in tissues is finding increasingly broad application as a new approach to histochemistry. One of the potential hazards in connection with all histochemical technics, including that of radioautography, is the leaching out or displacement of material under investigation which may occur as a result of tissue processing or staining. For example, studies at this laboratory[1] on radioautographic localization of $P^{32}$ in brain tissue have shown that marked losses of the tagged element are incurred in the course of fixing and staining for routine paraffin sections. Perhaps the surest way to avoid artifacts of this nature is to use completely unprocessed frozen material for the radioautograph exposure, omitting all fixing and staining until after the autograph has been completed. An advantage of the method to be described is that it not only makes such post-staining possible, but also makes the staining process quite independent of the radioautograph development. As a result, the tissue may be stained by any of the well-documented histochemical technics as adjunct to or control of the isotope localization. An outline of the technic and an example of its application has been described in a preliminary report from this laboratory.[2] Similar applications of stripping film have also been reported by Pelc, Boyd and others.[3] A more general discussion of radioautographic technics may be found in the review article by Evans[4] and the book of Yagoda.[5]

*Method. A. Films.* In a series of experiments[6] in which nineteen commercial emulsions were investigated with respect to sensitivity, contrast and grain size, Type M stripping film (now commercially available) was selected as best suited for our radioautograph technic on the basis of the following qualities: fine grain, high contrast, low background fog and thinness of emulsion. Emulsions with finer grain and consequently greater resolving power are available (as, for example, Ansco Reprolith ortho stripping film), but these are very much less sensitive and so call for exposures which are impracticable for most work. Type M stripping film represents a satisfactory compromise between the mutually exclusive extremes of fine grain and high sensitivity in commercial emulsions.

The emulsion of Type M stripping film is about 10 microns in thickness and is coated on a 7 micron transparent cellulose acetate stripping base. Emulsion and stripping base are in turn mounted on a transparent supporting base about 0.15 mm thick, as shown in Fig. 1. At the time of use the emulsion on its 7 micron base is stripped away from the supporting material and can then be handled almost as though it were an autonomous 10 micron layer of emulsion. To facilitate later steps, it is advantageous to have on hand pieces of film just large enough to overlap the edges of a standard microscopic slide.

B. Tissue Preparation. 1. Frozen sections. Our best histology has been obtained by freez-

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1 Steinberg, D., and Selverstone, B., unpublished data.

2 MacDonald, A. M., Cobb, Jock, and Solomon, A. K., *Science*, 1948, **107**, 550.

3 Pelc, S. R., *Nature*, 1947, **160**, 749; Boyd, G. A., and Williams, A. I., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 225; Endicott, K. M., and Clark, F. A. (Ref. 5, p. 48).

4 Evans, T. C., *Nucleonics*, 1948, **2**, 52.

5 Yagoda, H., Radioactive Measurements with Nuclear Emulsions, John Wiley and Sons, Inc., New York, 1949.

6 Cobb, Jock, and Solomon, A. K., *Rev. Sci. Inst.*, 1948, **19**, 441.


FIG 1.

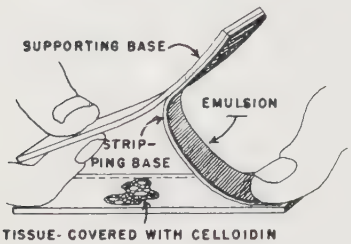


FIG 2.

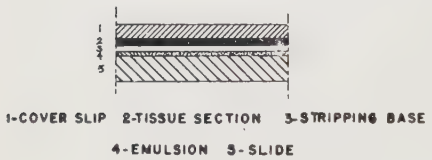


FIG 3.

ing the tissue to be studied immediately on removal in iso-pentane at liquid nitrogen temperatures, according to the technic of Linderstrøm-Lang and Mogensen.[7] It is then transferred to a refrigerated room at −10 to −15°C and cut on a microtome equilibrated at that temperature, making use of a modified Linderstrøm-Lang "window",[8] to prevent rolling of the sections. These are melted onto slides previously prepared by coating with 0.1% gelatin which is then hardened by dipping in 10% formalin. With this technic sections can be allowed to dry at room temperature without distortion. They are then ready for the application of the film in the manner described below.

2. Paraffin sections. When there is no problem of leaching out or displacement of the material under investigation, routine fixing, embedding and sectioning technics are employed. Needless to say, tissue detail obtained with this technic is decidedly superior to that of even the best frozen sections, and hence this technic is to be preferred whenever possible. The slides are stained in the usual manner up to the absolute alcohol stage. They are not carried through into xylol since the film is to be mounted with an alcohol base adhesive mixture. If the film cannot be mounted at the time staining is completed, the slides may be carried through to xylol, provided it is removed with absolute alcohol prior to mounting.

C. Application of the Film. All handling of film is carried out under Wratten Series 2 safelight. First, the emulsion and stripping base are separated from the heavy supporting base as shown in Fig. 1, leaving the last ¼ inch adhering to the base to facilitate manipulation. Stripping can usually be started at a corner of the film by stroking the emulsion away from the support with the ball of the thumb.§ Stripping should be done slowly to prevent any static discharge between stripping base and support, as such a discharge causes a considerable increase in background fog.

A 1% solution of celloidin in methyl alcohol is used to cement the film, *emulsion side up,* to the tissue. If the celloidin is dissolved in the conventional ether-alcohol mixture, irregular black splotches appear on the developed film. These artifacts have been eliminated by the use of methyl alcohol as solvent. The slides are laid tissue section up on a piece of filter paper and the alcohol from the paraffin sections is allowed to evaporate almost completely. Then a drop or two of the celloidin solution is placed directly on the tissue section and another drop on the slide to either side of the section. The partially stripped film is then applied to the section as shown in Fig. 2, and stripping of the film is completed at the same time. Next, the trans-

[7] Linderstrom-Lang, K., and Mogensen, K. R., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.,* 1938, **23**, 27.

[8] Coons, A. H., private communication.

§ To facilitate stripping, Dr. George A. Boyd[3] has suggested turning the film emulsion side down and slitting the supporting base with a razor blade. The backing can then be cracked along the slit. While this procedure simplifies stripping, it also increases the background fog due to the static discharge accompanying the breaking of the support. See Boyd's article for details.

parent supporting base is laid over the emulsion to serve as protection, and the preparation is smoothed with the fingers. Finally, a second microscope slide is placed on top of the supporting base and the entire "sandwich" wrapped with scotch tape. It is our practice to wrap each preparation individually in opaque paper and place it immediately under a pressure of 8 lbs/sq inch. Resolution is considerably improved by the more intimate tissue-film contact obtained with pressure. After 24 hours under weights the preparation is transferred to the refrigerating unit or dry ice box and stored at freezing temperature for the duration of the exposure time. Removal from under the weights at this time, after the celloidin has set, causes no apparent loss in resolution. Estimation of proper exposure times will be discussed below.

D. Developing and Mounting. Autographs ready for development are unwrapped in the darkroom and the scotch tape is cut along the edges of the "sandwich" with a razor blade. Unwinding the tape leads to a grossly visible blue static discharge which can fog the film quite badly. After removal of the guard slide and the supporting base, the bottom slide, bearing tissue and adherent stripping film, is placed in the developer *in toto*. It is carried as a unit through the stop bath and fixative. Each slide is developed in a separate test tube into which the developer and other solutions are successively poured.

In order to control the many variables that may affect film sensitivity we have adopted the following precautions as part of our standard procedure. (a) All films are kept in a desiccated box in the refrigerating unit. (b) Films are allowed to come to room temperature before use. (c) Exposures are all made at the same temperatures. (d) Films are developed immediately at the end of the exposure period. (e) Wratten Series 2 safelight at 3 feet is the only illumination used.

The developing process is controlled in the following ways. (a) Distilled water is used in all solutions. (b) Developer is stored in full, brown, stoppered bottles. (c) 50 cc of fresh developer is used for each preparation. (d) Each tube is inverted gently every 30 seconds during development. (e) All solutions are brought to 68° ± 0.5°C and kept at this temperature during development.

During the processing, the emulsion is directly and evenly exposed to the solutions, thus insuring complete, uniform development. The tissue is protected by the intervening stripping base and the celloidin coating, so that it is preserved from the action of the developing solution Since the stripping film gradually separates from the slide during processing, however, it is important to limit the time in the solutions. Five minutes in Kodak D-19 developer, 15 seconds in 1% acetic acid stop bath and two minutes in Kodak F-5 fixing-hardening solution constitutes a satisfactory routine procedure. After 5 to 10 minutes washing in cold water, the stripping film has become partially detached from the slide and a gentle, steady pull on one end suffices to complete the separation. The tissue section adheres to and comes away with the stripping film which can be left in a beaker of distilled water until it is mounted.

At this point we have the tissue section adherent to one side of the stripping base and on the opposite side the developed grains of the tissue's own radioautograph. We now simply take this preparation up through alcohols to xylol, and, after trimming away the excess film around the tissue, mount with balsam, or a substitute, and a cover slip. Usually, it does not matter whether the tissue is above or below the autograph, but when the image is very dense, the tissue detail is better examined with the tissue uppermost. The orientation of the final preparation is shown in Fig. 3.

At low magnification, the close juxtaposition of tissue with its autograph makes it possible to survey the preparation with a single fixed plane of focus. At high magnification, a slight change of microscope focus correlates the grain density pattern in the autograph with the histologic pattern.

E. Post-staining. When unstained sections have been used, the steps are exactly the same up to the point at which the preparation is removed from the wash water. In order to stain the tissue and at the same time to

protect the emulsion from the stain the stripping film is now cemented *emulsion side down* onto a clean slide with Kodalith Stripping Film Cement or with 1% celloidin in ether-alcohol. This leaves the tissue section exposed and the slide can be handled through the staining process in the routine manner. After staining, the excess film peripheral to the tissue section itself is trimmed away and the preparation covered with balsam, or a substitute, and a cover slip. In order to obtain a uniformly flat field it is necessary to weight the cover slip rather heavily until the balsam has dried.

F. Time of Exposure. Data have been presented elsewhere[9] that permit the calculation of exposure time for Type M stripping film for 5 important isotopes, $C^{14}$, $Ca^{45}$, $I^{131}$, $P^{32}$, and $Zn^{65}$, provided the activity of the preparation in terms of disintegration per minute per unit area is known. For problems in which such assay is inconvenient, a satisfactory empirical procedure has been developed, based on the use of Eastman No-Screen x-ray film, a film many times more sensitive than the Type M. Using this film, "trial" radioautograph preparations are made using tissue sections from the same block. The film is merely laid over the tissue section without adhesive, covered with a guard slide and "sandwiched" with scotch tape as above. At the time of development the "sandwich" is opened and the film removed and processed. Several such preparations are put up and developed at intervals. From the ratio of sensitivities of the No-Screen x-ray and Type M stripping films the exposure time needed to get an equivalent density with the latter can be calculated. Ratios of initial activity requirements from Table I indicate the relative sensitivities of the two films.

Table I (taken from reference 9) gives the activities required at the surface of the tissue to produce radioautographs of image density 0.1 and 0.6 above background with 15 day exposures to uniform sources of $C^{14}$, $Ca^{45}$, $P^{32}$, $I^{131}$ or $Zn^{65}$. It must be kept in mind that generally one is interested in some par-

[9] Steinberg, D., and Solomon, A. K., *Rev. Sci. Inst.*, in press.

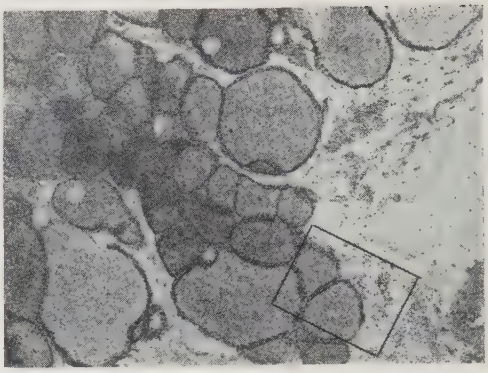

FIG. 4.

Radioautograph of rabbit thyroid. Animal was sacrificed 16 hours after intraperitoneal tracer dose of 270 microcuries $I^{131}$. Tissue was formalin fixed and stained with hematoxylin-eosin. Type M stripping film, exposed 16 hours, developed 5 minutes in Kodak D-19. Magnification 200×.

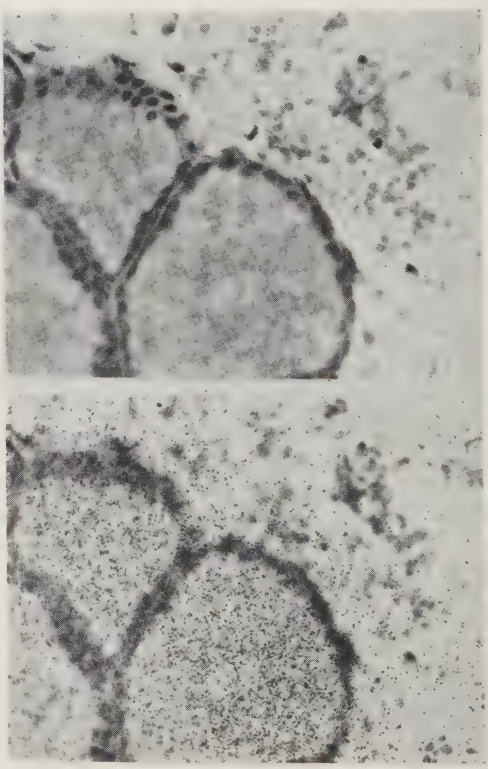

FIG. 5.

Enlargement of area blocked out in Fig. 4.

Upper: Focused in plane of tissue to bring out histologic detail.

Lower: Same area focused in plane of photographic grains for study of distribution and contrast. Magnification 800×.

TABLE I.
Initial Activity Required in Disintegrations per Minute per $cm^2$ of Tissue.

| | Eastman no-screen x-ray film | | Eastman type M stripping film | |
|---|---|---|---|---|
| | 0.1> Bkgd. | 0.6> Bkgd. | 0.1> Bkgd. | 0.6> Bkgd. |
| $C^{14}$ | 124 | 806 | 2,280 | 11,400 |
| $Ca^{45}$ | 124 | 1,030 | 3,260 | 27,000 |
| $I^{131}$ | 414 | 2,180 | 18,500 | 75,200 |
| $P^{32}$ | 843 | 4,610 | 36,700 | 160,000 |
| $Zn^{65}$ | 4,250 | 27,400 | 138,000 | 741,000 |

Values given assume a 15-day exposure and 5 minutes development in Kodak D-19. Data are for a uniform source, and so apply only to mean activity of tissue section.

ticular structure and so the average density of the autograph may be a very unreliable guide to optimum exposure. Both when counting the initial activity of tissue sections and when examining the images of preliminary No-Screen x-ray autographs, these areas of interest should where possible be the basis of estimation. It is absolutely essential that the precautions in handling of films and development described above be observed in order to obtain reproducible results.

*Discussion.* The advantages of the present technics are several:

1. The staining and developing processes are independent of one another.

2. The thickness of the emulsion is equal over the entire preparation so that differences in grain density at different points can be used as an index of the activity of the underlying point in the tissue.

3. Tissue and emulsion are held in a fixed relationship from the very beginning of the procedure through to the time of examination. Since one can look through both the autograph and the tissue simultaneously under the microscope, correlation and interpretation are made simple.

One limitation of the technic lies in the 7 micron separation of tissue and emulsion. While in many studies this very small separation may not be of importance, it does limit the theoretical resolution possible with the technic. However, our results with Type M stripping film indicate that resolution to 10-15 microns can be obtained, as illustrated by Fig. 4 and 5.

These autographs show the very different rates of uptake of $I^{131}$ by different thyroid follicles. The fine grain of the emulsion can be noted and the order of magnitude of the background can be estimated from the areas of emulsion not overlying tissue. At 16 hours after an injection of iodide the material is known to be widely disseminated throughout the gland and this is evidenced in the autographs. Of particular interest is the rather sharp demarcation of the high activity colloid material from the low activity thyroid stroma.

The data of Table I for Type M stripping film should make clear a limitation of the radioautographic technic that is too seldom emphasized, the need for high tissue activity. Unless the material under investigation is highly concentrated by the organism as in the case, say of radioiodine, the specific activity requirements are high and in some cases forbiddingly high. With the aid of data on exposure, such as that referred to above, it is often possible to determine beforehand the feasibility of a study when the specific activity of the material and the probable biologic dilution are known.

*Summary.* A technic for radioautography is described which makes the developing and staining processes independent and, through the use of stripping film, provides an emulsion of uniform thickness. The method of mounting permits uniform development of the entire emulsion and maintains a fixed tissue-autograph relationship.

We should like to express our thanks to Dr. A. H. Coons for advice and assistance in the technic of cutting frozen sections. One of us (A.M.M.) has held a Rockefeller Fellowship in Pathology. This work has been supported in part by the Office of Naval Research and the Atomic Energy Commission.

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# Failure of Antihistaminic Drug "Phenergan" to Protect Against Acute Pulmonary Edema. (17351)

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Halpern and his co-workers have developed the thesis that antihistaminic drugs owe much of their therapeutic effectiveness to a primary influence upon cellular permeability, aside from their property of competitive inhibition of histamine. This concept is based in part on the observation that N-($\beta$-dimethylamino-$\alpha$-methylethyl)-phenothiazine (3277 R.P. or Phenergan) protects guinea pigs and rabbits from pulmonary edema produced by an overdose of epinephrine[1,2] or by certain poison gases, such as chloropicrin.[3]

On the other hand, Stone and Loew[4] failed to obtain protection by pyranisamine (Neo-Antergan) in rabbits with epinephrine-induced pulmonary edema. The same authors[5] also could not confirm the observation that Phenergan protected rabbits. In one series (8 animals) they obtained a reduced mortality but no reduction in pulmonary edema, as judged by lung weight. It seemed of interest to determine whether Halpern's results could be confirmed in our laboratory, and also whether Phenergan would affect pulmonary edema produced by administration of ammonium salts, as described by Koenig and Koenig.[6] If it could be shown that pulmonary edema produced by two such widely different means could be alleviated by an antihistaminic drug, support would be added to Halpern's view that such drugs may primarily affect cellular permeability.

*Methods.* The rats used in this study weighed about 200 g. Both Carworth and Holtzmann rats were employed, and as no difference was noted in results obtained with the two strains, they have been combined in the summary. The guinea pigs varied from 300 to 500 g in weight. Pulmonary edema was produced as follows: epinephrine chloride was injected in the forelimb vein of guinea pigs, 0.6 mg per kg (the dose used by Reuse[3]); ammonium chloride was administered intraperitoneally, the dose being 400 mg per kg for rats, and 600 mg per kg for guinea pigs, as described by Koenig and Koenig.[6]

Phenergan was chosen as the antihistaminic drug, because Halpern has stated that its effect on cellular permeability was greater than any others he used. We administered Phenergan subcutaneously, except to a few of the rats, where the intraperitoneal route was employed. The dose was the same as that used by Halpern and by Reuse; namely, 20 mg per kg, except in 2 groups of rats where the dose was doubled. Thirty to 60 minutes (usually the latter) were allowed to elapse between injection of Phenergan and of epinephrine or ammonium chloride. The lot of Phenergan used was manufactured in France, and obtained from Societé des Usines Chimiques Rhone-Poulenc, Paris.

*Results.* The syndrome of acute experimental pulmonary edema in rats and guinea pigs has been adequately described by Koenig and Koenig.[6] The signs and gross findings at autopsy described by them were seen in the majority of our animals.

The results are summarized in Table I. It is evident that Phenergan, even in the relatively large doses used, did not protect the animals from pulmonary edema as judged by lung weight, nor did the antihistaminic drug reduce the mortality, nor prolong the survival

[1] Halpern, B. N., Hamburger, J., and Cruchaud, S., *Acta allerg.*, 1948, **1**, 97; Halpern, B. N., and Cruchaud, S., *Compt. rend. soc. biol.*, 1947, **141**, 1038; Vermeil, G., Halpern, B. N., and Cruchaud, S., *Compt. rend. soc. biol.*, 1949, **143**, 77.

[2] Reuse, J., *Compt. rend. soc. biol.*, 1948, **142**, 638.

[3] Halpern, B. N., and Cruchaud, S., *Compt. rend. Acad. d. sc.*, 1947, **225**, 1194.

[4] Stone, C. A., and Loew, E. R., *Fed. Proc.*, 1949, **8**, 335.

[5] Stone, C. A., and Loew, E. R., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 122.

[6] Koenig, H., and Koenig, R., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 375.

TABLE I.
Lack of Protective Effect of Phenergan Against Acute Pulmonary Edema Produced by Epinephrine or Ammonium Chloride.

| Exp. No. | Treatment | Avg body wt, g | Dose of phenergan, mg/kg | Mortality ratio* | Median survival time,† min. | Avg lung wt,‡ g/kg body wt |
|---|---|---|---|---|---|---|
| | | Rats | | | | |
| I | $NH_4Cl$ | 199 | — | 8/15 | 35 | 11.6 |
| II | " + phenergan | 220 | 20 | 4/10 | 20 | 18.2 |
| III | " + " | 206 | 40 | 8/10 | 24 | 14.6 |
| IV | Phenergan | 196 | 40 | 0/10 | — | 6.2 |
| V | No drug | 212 | — | 0/4 | — | 7.7 |
| | | Guinea pigs | | | | |
| VI | $NH_4Cl$ | 461 | — | 8/10 | 8 | 10.4 |
| VII | " + phenergan | 408 | 20 | 12/12 | 8 | 8.6 |
| VIII | Epinephrine | 436 | — | 6/7 | 7 | 19.7 |
| IX | Epinephrine + phenergan | 423 | 20 | 8/8 | 7 | 20.3 |
| X | No drug | 450 | — | 0/5 | — | 6.8 |

* Mortality ratio = number dead/number used.
† Excluding those surviving indefinitely.
‡ Avg lung wt in experimental groups include only those animals which succumbed after injection. Control animals were sacrificed under pentobarbital anesthesia.

time of the animals which died. The difference in lung weight between Groups VI and VII is not significant ($P > 0.05$), but Group VII differs significantly from control Group X ($P = 0.02$), as does Group VI ($P < 0.01$). In all the other cases, the differences between experimental and control groups are obvious from inspection of the table.

*Discussion.* The lung weights of the guinea pigs succumbing to epinephrine were greater than in those receiving a lethal dose of ammonium chloride. It may therefore be assumed that pulmonary edema was not maximal in the latter group, and may not have been the sole cause of death. Stone and Loew[5] have also expressed the view that pulmonary edema may not have been the sole cause of death in their epinephrine treated rabbits. The overall mortality rate in all our animals receiving edema-producing drug without Phenergan was 22/32 or 69%, while in those receiving Phenergan in addition to the toxic agent it was 32/40 or 80%. Our results are in agreement with the statement of Stone and Loew,[5] that Phenergan did not protect the animals against pulmonary edema, although we did not observe the lower mortality rate which they reported in their animals.

Koenig and Koenig[6] believe that the pulmonary edema produced by ammonium chloride is a specific action of the ammonium moiety, and is not related to the acidosis produced by $NH_4Cl$. Other ammonium salts also induced pulmonary edema, while acidosis produced by other means did not. However, they did not publish data indicating the degree of acidosis produced by the various agents they used, and it is therefore impossible to judge the validity of their conclusions.

The divergence between our results and those of Reuse[3] is not easy to explain. He used guinea pigs about twice as large as those herein reported. He also used epinephrine ascorbate, while the epinephrine we used was Adrenalin chloride (Parke, Davis), a glandular extract containing an unknown amount of nor-epinephrine. Halpern[1] stated that Phenergan did not prevent the blood pressure and cardiac effects of epinephrine, but Reuse[7] found that Phenergan in large doses inhibited both the pressor effect and the apnea produced by small doses of epinephrine. This effect, however, lasted only a few minutes, and probably does not explain the partial protection which he observed. Even in Reuse's experiments, Phenergan did not offer complete protection against epinephrine-induced pulmonary edema, as the

[7] Reuse, J., *Ann. soc. roy. d. sciences med. et nat. d. Bruxelles,* t. II, 1949.

mortality ratio was 5/14 in the Phenergan-treated animals, compared to 11/14 in those receiving 0.55 - 0.6 mg per kg of epinephrine without Phenergan. In the group to which 1.2 - 1.5 mg per kg of epinephrine was administered, 8 of 11 Phenergan-treated animals died.

Our results, as well as those of Stone and Loew, do not lend support to Halpern's hypothesis that Phenergan opposes the formation of pulmonary edema by virtue of the property of inhibiting cellular permeability.

*Summary.* The antihistaminic drug, Phenergan (3277 R.P.), in doses of 20 or 40 mg per kg, failed to protect rats or guinea pigs against the pulmonary edema induced by injection of ammonium chloride, and also offered no protection against epinephrine-induced pulmonary edema in guinea pigs.

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## Maintenance of Spontaneous Activity within the Cerebellum.*† (17352)

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The hitherto unsuspected widespread projection of the cerebellum[1] to the cerebral cortex and the ease with which facilitation[2] and suppression[3] of cortically and reflexly induced movements can be produced by cerebellar excitation emphasizes the need to answer the interesting but intricate question, what is the cerebellar influence on the rest of the nervous system? One approach to an answer is the study of mechanisms underlying the maintenance of the fast electrical activity of the cerebellum. The best study yet made on the nature of this electrical activity was that by Dow[4] who not only described it in great detail, but also studied the influence of various drugs on it and related the activity to muscular tone.

In the present study we have confirmed many of Dow's observations and extended the problem into an analysis of intrinsic cerebellar mechanisms which maintain what is probably the fastest electrical activity within the nervous system.

*Methods.* In mature cats, decerebrated, or placed under ether, nembutal, dial, chloralosane, or various combinations of the above mentioned anesthetics, the calvarium was removed to expose all parts of the cerebellum except the anterior one-half of anterior lobe and paraflocculus and flocculo-nodular lobe. Bipolar and monopolar silver wire as well as saline-cotton wick pick-up electrodes were used to introduce the cerebellar activity into Grass Model 111 amplifiers. The amplified electrical activity was observed and photographed on a five-inch cathode ray tube.

*Results.* The first question was: Does this fast electrical activity result from the driving of the cerebellum by extrinsic centers or is it something intrinsic to the cerebellum? As shown in Fig. la, the activity results from intrinsic mechanisms within the cerebellum. The electrical activity in this preparation was normal despite the fact that 5 days previous all afferent and efferent fibers (verified histologically) had been cut. Previously, Dow[4] reported fast electrical activity following "acute incomplete section of the cerebellar peduncles in four experiments" and Spiegel[5] also observed it

Further support for this observation is given

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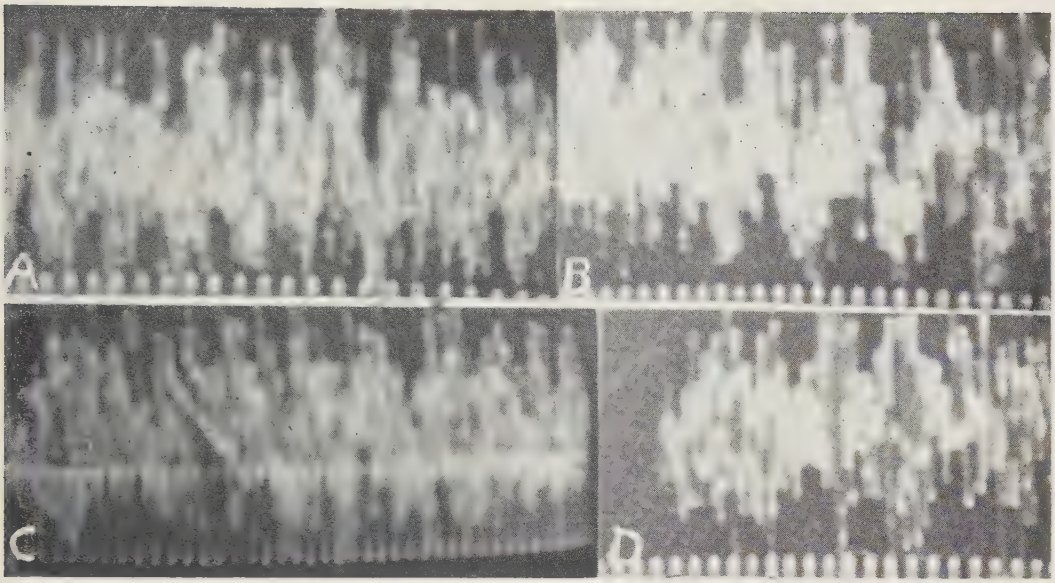


FIG. 1.

Electrical activity of the cerebellum recorded from the pial surface with bipolar silver wire pick-up electrodes. 100/sec. time signal.

A. Cat under nembutal anesthesia. Five days previously all 6 cerebellar peduncles had been transected, thus interrupting all afferent and efferent fibers. Note normal activity.

B. Cat under nembutal anesthesia. Note electrical activity in cerebellar cortex dissected free of animal and lying on saline moistened gauze. Nuclear cells were not present.

C. Cat under nembutal anesthesia. Note "normal" electrical activity, despite the total bilateral destruction of cerebellar nuclei.

D. Cat under nembutal anesthesia. Note electrical activity similar to A, B, C, above. Cerebellar peduncles and cerebellar nuclei left intact.

in Fig. 1b which shows the electrical activity in a part of the cerebellum which was dissected free and was lying on a strip of gauze several centimeters from the animal. Such activity, however, does not continue for more than 2-4 minutes

Since the fast electrical activity can continue in the absence of all fiber connections with the rest of the nervous system, then it must be maintained by intrinsic mechanism within the cerebellum.

The next question was: What are the intrinsic mechanisms which maintain this electrical activity? The cerebellar cortex is an ideal preparation with which to work because the anatomy is simple, well known, and remarkably uniform in structure. Fig. 2 summarizes the present state of our information on the anatomy of this organ. Working on the assumption that this activity is maintained either by reverberating circuits, or spontaneous activity of cellular aggregates, or both, the following analysis has been made:

*Reverberating Circuits.* A glance at Fig. 2 indicates the possibility of two reverberating circuits: 1. Purkinje cell (A) to central cerebellar nuclei, (B) back to Purkinje cell. That this circuit cannot be the responsible one is shown by the experiment illustrated in Fig. 1c. Note that the activity appears normal despite the absence of all the central nuclei on both sides.

A second possible reverberating circuit is Purkinje cell (A) through Purkinje cell axon collateral (I) back to a second Purkinje cell. Thus, one Purkinje cell could fire another, then a third, etc., and back again. Although our experiments do not disprove the possibility of this circuit functioning in this manner, as can be seen below, there are other more tenable explanations.

*Spontaneous activity of cellular aggregates.* The most conspicuous cell in the cerebellar cortex is the Purkinje cell. The large soma,

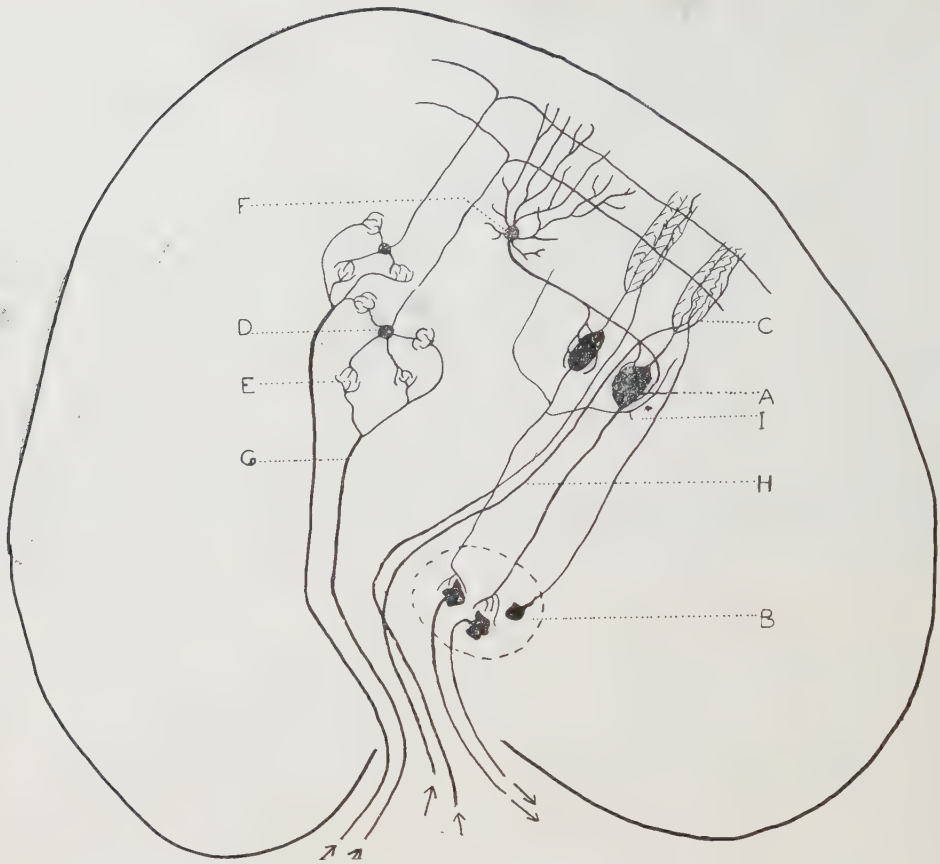


FIG. 2.

Diagrammatic drawing of microscopic anatomy of cerebellum. Modified from Ranson, 1935, The Anatomy of the Nervous System. A, Purkinje cell body. B, Cell body in central cerebellar nuclei. C, Purkinje cell dendrites. D, Granule cell body. E, Glomerulus in which afferent fiber mossy terminal synapses with granule cell dendrite. F, Basket cell body. G, Afferent fiber terminating as mossy terminal. H, Incoming fiber terminating as climbing fiber. I, Purkinje cell axon collateral.

the elaborate branching dendrites, and the axonal connections to the central nuclei make it one of the important cells, and one of the first to be considered as a possible source of spontaneous activity. This cell is of further interest because of the spatial representation of other cellular components on its cell body and processes. The basket cell axons synapse around the cell body, while the granule cell (Fig. 2, D) axons and climbing fibers (Fig. 2, H) synapse on the Purkinje cell dendrites. Possible synapse points on Purkinje cell axons are not known. As is well known, granule cell axons connect with Purkinje cells along the longitudinal axis of the folium and basket cell axons connect with them along the transverse axis of the folium. In this manner, impulses coming from granule cells can be widely dispersed in the longitudinal and transverse planes of the cerebellar cortex and, from Purkinje cells, can be relayed in turn to the central cerebellar nuclei thence to centers within the brain stem. Thus, one cannot doubt the major role played by these cells in relaying the message through the cerebellar cortex. However, in order to study the role played by these cells in the maintenance of the fast electrical activity, according to the technics we were using, it would be desirable to selectively destroy them, leaving intact the other

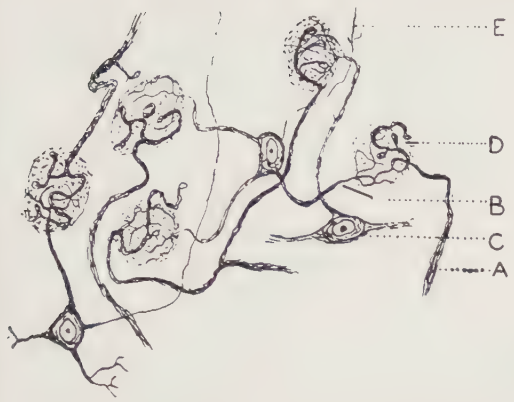


FIG. 3.
Outline drawing of microscopic anatomy of granule cell and granule cell glomerulus (modified from Cajal). A, Afferent fiber terminating as mossy fiber in protoplasmic glomerulus, D. C, Granule cell body. B, Dendrite of granule cell synapsing with afferent fiber in granule cell glomerulus. E, Granule cell axon.

cellular elements of the cerebellar cortex. We were not able to accomplish this technical procedure.

The Purkinje cell is not the only potential source of cellular elements for the maintenance of spontaneous electrical activity. A second possible source is the granule-basket cell combination in which the granule cell glomerulus deserves some comment. This non-nucleated protoplasmic structure located at the junction of the incoming mossy terminal with the dendritic terminals of the granule cells is approximately 15 $\mu$ in diameter. Fig. 3 illustrates the salient properties of the various parts of the granule cell. Note that the incoming afferent fiber (A) synapses within the glomerulus (D) on the way to the granule cell (C) dendrite (B). Note also that, except for a slightly granulated appearance, the protoplasm appears almost structureless.

To check the possibility of activity coming from the granule cell layer, we attempted to record directly from this layer with pick-up wire (26 gauge, insulated to tip). Although good activity could be recorded, and often better activity than that from the pial surface, which is approximately 250 $\mu$ from the granular layer, the answer is still equivocal because possible activity of nearby Purkinje cells may have spread to the needle tip or Purkinje cell axons passing nearby may have been conducting activity through the area to the underlying cerebellar nuclei. From this, one must conclude that if the intrinsic spontaneous electrical activity of the cerebellar cortex results from spontaneous activity of cellular aggregates, as seems likely, then the basket-granule cell Fig. 2 D,F (with glomerulus) combination appears to be a possible responsible agent. One thing is certain, either it is this cellular complex or it is the Purkinje cell itself which is responsible.

*Summary.* Evidence is presented to show: (a) that the fast electrical activity of the cerebellum is intrinsically maintained within this structure; (b) that it is not due to reverberating circuits through the cerebellar nuclei and back to the cerebellar cortex; (c) that it is probably due to the spontaneous activity of Purkinje cells and/or the granule-basket cell combination.

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## Prevention of Secondary Infection due to *Pseudomonas aeruginosa* in Frostbitten Tissue. (17353)

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One of the major problems encountered in investigations on experimental frostbite is the prevention of infection of the frozen tissue. Without prophylactic treatment infection occurs in practically all cases of severe frostbite. The following report gives the results of the application of penicillin and sulfamylon*

* The sulfamylon hydrochloride powder was supplied by the Medical Research Department of Winthrop-Stearns, Inc.

TABLE I.
Incidence of Infection in Frostbitten Legs Treated Locally with Penicillin.

| | Number | Not infected | Infected |
|---|---|---|---|
| Heparin treated | 42 | 32 | 10 |
| Non-heparin treated | 36 | 16 | 20 |
| Total | 78 | 48 | 30 |

$\chi^2 = 6.82$ and $P = 0.008$.

heparin therapy and 128 did not. The occurrence of gangrene was practically the same in the two series. Of the entire group 48 saved the frostbitten leg completely, 42 lost part of the injured limb, 105 lost the leg completely, and 95 died, 14 of them after the final fate of the leg was decided.

*Penicillin.* Seventy-eight animals, heparinized and non-heparinized, received local treatment with penicillin ointment (1,000 units per

TABLE II.
Incidence of Gangrene in Infected and Non-infected Animals Treated with Penicillin.

| | Number | Completely saved | Partially saved | Completely lost | Died |
|---|---|---|---|---|---|
| Non-infected | 48 | 0 | 11 | 18 (2)* | 19 |
| Infected | 30 | 0 | 4 | 22 | 4 |

* Number in parentheses indicates animals which died after the fate of the leg was decided.
$\chi^2 = 2.00$ and $P = 0.15$.

(p-aminomethylbenzenesulfonamide) to the injured area after severe experimental local cold injury.

The experiments were performed on 290 male white albino rabbits weighing 2,000 to 3,000 g. The injury was produced by immersing one depilated hind leg for 30 minutes in an alcohol bath cooled with dry ice to temperatures ranging from −10°C to −25°C. The exact experimental procedure is described in another paper.[12] After the experiment the frozen area was covered with wool fat and a sterile dressing applied. During the first week the dressings were changed every day, sometimes every other day. Infections usually became evident between the fourth and sixth day after injury. The infected frozen area appeared bluish green, swollen, and slightly translucent. Small hemorrhages in the parts bordering the healthy tissue were quite often present. Pus oozed from the injured area, especially from the region of the demarcation line. From the bluish discoloration of the bandages and from the characteristic odor it was assumed that the infection was caused by *Bacillus pyocyaneus.* The bacteriological examination of 2 cases revealed pure cultures of *Pseudomonas aeruginosa (B. pyocyaneus).* The clinical appearance was characteristic enough that no further bacteriological investigation was considered necessary.

Of 290 animals 162 received intravenous cc). In this group 30 animals showed signs of infection in the frostbitten area and 48 did not.

The degree of injury was the same in both groups of animals. The difference in the incidence of infection in the heparinized and non-heparinized animals is significant ($\chi^2 = 6.82$ and $P = 0.008$). We do not have an explanation for this finding. We do not believe the evidence sufficient to support the conclusion that heparin is effective in preventing local infection of frostbitten tissue. The incidence of gangrene of the frostbitten legs in the infected and non-infected animals shows no essential differences (Table II). The higher death rate in the non-infected group is mainly due to the fact that most of the deaths occurred before the fourth or fifth day when the infections usually became evident. The relation of partially lost to completely lost legs is slightly less favorable for the infected animals ($\chi^2 = 2.00$ and $P = 0.15$). But even if this difference were significant it would not necessarily mean that it was due to the infection. According to our observations the occurrence of infection of frostbitten tissue in rabbits is related to the diarrhea that develops in some animals after cold injury. There seems to be a higher probability for the appearance of diarrhea with increasing severity of cold injury.

*Sulfamylon.* One hundred and eighty ani-

TABLE III.
Comparison of the Effect of Local Treatment with Penicillin and with Sulfamylon.

| | Number | Not infected | Infected |
|---|---|---|---|
| Penicillin treated | 78 | 48 | 30 |
| Sulfamylon treated | 212 | 208 | 4 |
| Total | 290 | 256 | 34 |

$\chi^2 = 70.2$ and $P = <0.0001$.

mals were treated in exactly the same manner as the preceding group, except that instead of penicillin ointment an ointment with 3% sulfamylon was used. Of these animals 60 did not receive any other treatment while 120 were heparinized. Signs of local infection did not occur in these animals.

Another group of 32 rabbits was frostbitten in the same manner as in the preceding series. They were used for temperature measurements during freezing and thawing and received little care thereafter. They were routinely treated by local application of sulfamylon ointment, but the dressings were usually changed every second to fourth day. In this group 4 animals developed local infection at the site of injury in spite of the application of sulfamylon. Remarkably enough these infections were found after the dressings had not been checked for 3 days.

Even if we include the separate group of 32 animals with inadequate treatment, in which the only infections during sulfamylon treatment occurred, the results show undoubtedly the superiority of sulfamylon.

The statistical computation of these results shows that the difference is highly significant ($\chi^2 = 70.2$ and $P = <0.0001$).

*Discussion.* Since the introduction of p-aminomethylbenzenesulfonamide (marfanil or sulfamylon) it has been shown to be a very effective drug in the prevention and cure of local infection. It is effective against a most diversified number of organisms in clinical as well as laboratory tests.[1,3,5–10,13] Its effectiveness against *Pseudomonas aeruginosa (B. pyocyaneus)* which caused the infections in our frostbite experiments is of special interest. Clinical infections with *Pseudomonas aeruginosa* respond well to treatment with p-aminomethylbenzenesulfonamide.[2,5,6,9] The same results were obtained in bacterial culture tests.[6,8] We agree with Howes[6] that this drug is distinctly superior to penicillin in its effectiveness against *Pseudomonas aeruginosa.* But since the effect is bacteriostatic,[6,8] it seems necessary to continue the treatment regularly until the possibility of infection has passed. The usefulness of p-aminomethylbenzenesulfonamide in the prevention and cure of infection in frostbite has already been reported in former papers.[11,14] It is also effective in the treatment of deep burns[4] where similar tissue conditions exist.

*Summary and conclusions.* 1. Of 78 animals with severe experimental frostbite 30 cases of infection with *Pseudomonas aeruginosa (B. pyocyaneus)* occurred, although the frozen area was treated locally with penicillin ointment.

2. Of 212 animals with the same degree of cold injury and treated with 3% sulfamylon ointment, only 4 animals developed local infection with *Pseudomonas aeruginosa.* In 180 of these animals the dressings were changed daily and none became infected. The dressings of 32 animals were changed every 2 to 4 days; in this group 4 infections occurred.

3. Sulfamylon is superior to penicillin in its effect on *Pseudomonas aeruginosa* infections.

4. It is possible to control the infection due

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to *Pseudomonas aeruginosa* in experimental frostbite by daily local application of sulfamylon.

We wish to give credit to Mr. Allyn Kimball of the Department of Biometrics, School of Aviation Medicine, Randolph Field, Texas, for making the statistical analyses of the experimental data.

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## Use of Heparin in Treatment of Experimental Frostbite. (17354)

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During World War II casualties from local cold injury were numerous. In some units they exceeded the number of casualties from every other cause. This was especially true of Air Force personnel on high altitude bombing missions. It was therefore quite natural that much interest was focused on the investigation of the mechanism and therapeusis of cold injury.

Among the publications on the treatment of local frostbite, the reports of Lange and his collaborators were the most remarkable.[1–5] These authors described the results of heparin in the prevention of gangrene in animals and human volunteers after local cold injury. The control cases in their experiments always developed extensive or complete necrosis of the frostbitten area whereas the heparin treated cases almost always escaped gangrene. The details of the experimental procedures and of heparin administration as used in their investigations will be discussed later.

Publications subsequent to those of Lange *et al.* did not confirm their results.[6,7,8] Because of the importance of the subject to the Air Force we deemed it advisable to repeat these experiments.

*Material and Method.* With few exceptions in the early series we used white male albino rabbits of more than 2,000 g body weight. Female animals were excluded since in one series they seemed to be more susceptible than males to internal hemorrhages after heparin administration. The animals were kept in the open air throughout the year, but at least one week before the experiment they were brought into the air conditioned animal house and kept at 25°C (77°F). The food consisted of Purina rabbit pellets and occasionally carrots; water was unlimited. In the course of the investigation during which more than 300 animals passed through our animal room only 2 animals died spontaneously.

During the first part of the experiment we followed the procedure of Lange *et al.*[2,5] as closely as possible, except that the animals were not anesthetized during freezing. The right hind leg was clipped and depilated with Zip Depilatory Cream from the ankle upwards and loosely covered with a rubber condom boot to about 2 cm to 3 cm above the knee joint. The prepared hind leg was immersed to the knee joint in a bath at –25°C (–13°F) for 30 minutes. The freezing bath consisted of 95% alcohol cooled with dry ice. After some experience it was possible to keep the bath within 0.5°C of the desired temperature. Immediately after freezing, the legs were

1 Friedman, N. B., Lange, K., and Weiner, D., *Am. J. M. Sc.*, 1947, **213**, 61.

2 Lange, K., and Boyd, L. J., *Surg., Gynec., and Obst.*, 1945, **80**, 346.

3 Lange, K., and Loewe, L., *Surg., Gynec., and Obst.*, 1946, **82**, 256.

4 Lange, K., Boyd, L. J., and Loewe, L., *Science*, 1945, **102**, 151.

5 Lange, K., Weiner, D., and Boyd, L. J., *New England J. Med.*, 1947, **237**, 383.

6 Fuhrman, F. A., and Crismon, J. M., *J. Clin. Invest.*, 1948, **27**, 364.

7 Quintanilla, R., Krusen, F. H., and Essex, H. E., *Am. J. Physiol.*, 1947, **149**, 149.

8 Schumacker, H. B., Jr., White, B. H., Wrenn, E. L., Cordell, A. R., and Sanford, T. F., *Surgery*, 1947, **22**, 900.

TABLE I.
Results of Heparin Treatment in Very Severe Frostbite.

| No. of animals | Treatment | No necrosis | Partial necrosis | Complete necrosis | Died | Survived |
|---|---|---|---|---|---|---|
| 12 | 2 cc heparin every 4 hr | 0 | 3 | 2 | 7 | 5 |
| 30 | 3 cc heparin every 6 hr | 0 | 4 | 23 (1)* | 4 | 26 |
| 39 | 3 cc heparin every 12 hr | 0 | 1 | 12 | 17 | 22 |
| Total—81 | | 0 | 8 | 46 (1)* | 28 | 53 |
| Controls—42 | | 0 | 7 | 28 (2)* | 9 | 33 |

* Number in parentheses indicates animals which died after fate of the leg was decided and which therefore appear twice in the table.

123 animals frozen in an alcohol bath at —25°C with rubber boot loosely attached to leg.

81 animals were treated with heparin for 6 days.

42 animals were controls.

The distribution of "partial necrosis" and "complete necrosis" is essentially the same in both groups. ($\chi^2 = 1.2$ and $P = 0.27$).

The death rate is not significantly different. ($\chi^2 = 1.7$ and $P = 0.18$).

covered with sterile dressings and the animals put into individual cages. Generally the dressings were changed every day. The animals which showed severe swelling had the first dressing changed after 12 hours to prevent obstruction of the circulation by a tight bandage. At the end of the first day the swelling was at its peak, and thereafter the danger from constrictive dressings was negligible. We originally used a thin layer of wool fat to prevent adherence of the bandages due to the oozing of plasma. After some infections occurred, we added penicillin to the wool fat, but without success. Subsequently sulfamylon was used which eliminated further infection in the course of the investigation. The prevention of infection following experimental frostbite is the subject of a separate paper.[9]

In the second part of the investigation the conditions of exposure were changed since we felt it necessary to use a less severe cold injury to evaluate heparin therapy.

The experiments were performed from February 1948 to March 1949 on 315 animals. Of these, 153 animals received heparin* for 6 days, 80 served as controls, and 82 were used for the determination of a degree of injury suitable for the evaluation of the treatment.

The statistical method used is a test of significance using $\chi^2$ (chi-square) in two-way contingency tables.

*Repetition of the Experiments of Lange and Collaborators.* Lange and his co-workers used different temperatures and exposure times in their animal experiments.[2,5] The largest group with homogeneous conditions consisted of 41 animals of which one hind leg was exposed to a bath of –30°C (–22°F) for 30 minutes.[5] Twenty-one of these animals received 3 cc of heparin intravenously every 12 hours for 6 days. In a personal communication Lange advised us to give 3 cc of heparin every 6 hours.

The corresponding group in our experiments consisted of 123 animals. One hind leg was depilated, loosely covered with a rubber boot, and immersed to the knee joint in a bath of –25°C (–13°F) for 30 minutes and then allowed to thaw at room temperature. Eighty-one of these animals received heparin and 42 served as controls. The heparin was administered by 3 different regimes as shown in Table I. The initial injection of heparin was made from 1 to 4 hours after freezing in 51 animals, 4 to 8 hours in 14, and 8 to 12 hours in 16.

Blood coagulation times were determined

9 Pichotka, J., and Lewis, R. B., to be published.

* The heparin used was liquaemin (Roche-Organon, Inc.) and heparin (Abbot).

in 28 experiments following the injection of 2 cc of heparin intravenously. The capillary method was used; blood samples were obtained from the ear veins by means of nicking with a sharp needle. Intervals between determinations were 10 minutes in one series and 30 minutes in another.

The results of this experiment do not show any benefit of heparin treatment. In both groups all animals developed tissue necrosis. The statistical evaluation of the observed frequencies of *partial and total necrosis* in *treated and untreated animals* shows a value for $\chi^2$ of 1.2 and for P of 0.27. Hence the results in the two groups are not significantly different. The death rate for the heparin treated animals is slightly greater than for the controls but the difference is not significant ($\chi^2 = 1.7$ and $P = 0.18$). Table I.

*Use of Heparin in Less Severe Frostbite.* Since our results were not in agreement with those of Lange *et al.* we sought reasons for the discrepancy. The most probable explanation for the differences seemed to be that we did not produce the same degree of injury in our experiment that Lange had in his. Therefore, we first had to standardize our method of producing cold injury. The use of the rubber boot as described by Lange is a possible source of error. Fixation by longitudinal strips allows a considerable amount of air to remain between the leg and rubber boot, which might vary considerably in amount with different sizes, shapes, or positions of the legs. The heat conductivity is considerably altered by the layer of air around the leg and the degree of this change depends on the quantity of air in the boot.

For practical reasons experiments on frostbite with short exposure times must be performed in a fluid with a low freezing point, such as alcohol. Alcohol has an injurious effect on the skin as we could observe in our experiments. Therefore, it is not possible to escape the use of a protective covering which would have been the simplest way to avoid difficulties from altered conductivity.

We overcame this difficulty by attaching the rubber boot as closely to the leg as possible. After the boot was pulled over the foot and fixed loosely with circular adhesive strips at the ankle, the air was pressed out of the boot. The entire depilated limb was then covered with a thin layer of wool fat and the rubber boot pulled over the leg thus making it adherent to the skin. Finally the boot was fixed above the knee by a broad circular strip of tape and at the ankle with a narrow adhesive band. Folds in the rubber boot which sometimes could not be avoided were moved to the posterior aspect of the leg.

The legs of animals prepared in this way were immersed for 30 minutes in a bath of from 0°C to –25°C (+32°F to –13°F) with intervals of 5°C. The actual temperatures in different depths of the exposed leg were recorded simultaneously throughout the exposure and period of thawing.[10]

Judging from the clinical results and from the recorded temperatures inside the leg, the experiments with the rubber boot closely adherent were reproducible to a high degree.

For an exposure time of 30 minutes the temperature range of the freezing bath between –10°C and –15°C (+14°F to +5°F) was of greatest interest (Table II). At –10°C (+14°F) 25 of 26 animals did not develop necrosis and one showed superficial gangrene of the frozen limb; at –15°C (+5°F) 4 animals of 38 showed no necrosis, 16 partial necrosis, and 12 complete loss of the frozen leg. Six died before the fate of the frozen leg was decided. This distribution indicates that the injury of the group exposed at –15°C (+5°F) was just past the borderline from which spontaneous recovery was not possible for the overwhelming majority of our animals. We therefore assumed that any beneficial effect of heparin in the prevention of gangrene after frostbite would become evident under these conditions.

The actual temperatures in the deep muscle recorded in this group during exposure are much higher than in those immersed for 30 minutes at –25°C. On the average they barely passed the range of –6° to –7°C considered by Lake[11] to be decisive for the occurrence of

[10] Pichotka, J., and Lewis, R. B., to be published.

[11] Lake, N. C., *Lancet*, 1917, **2**, 557.

TABLE II.
Results from Exposure for 30 Minutes at Different Temperatures without Treatment.

| Bath temp. | Avg deep muscle temp. | No necrosis | Partial necrosis | Complete necrosis | Died | No. animals |
|---|---|---|---|---|---|---|
| —10°C | —3.3°C | 25 | 1 | 0 | 0 | 26 |
| —15°C | —7.4°C | 4 | 16 (1) | 12 (1) | 8 | 38 |

TABLE III.
Results of Heparin Treatment in Less Severe Frostbite.

| No. animals | No necrosis | Partial necrosis | Complete necrosis | Died | Survived |
|---|---|---|---|---|---|
| Treated—72 | 3 (2) | 21 (6) | 22 (12) | 46 | 26 |
| Controls—38 | 4 | 16 (1) | 12 (1) | 8 | 30 |

110 animals exposed at —15°C for 30 minutes with rubber boot adherent to the leg.
72 animals received 3 cc of heparin every 6 hours for 6 days.
38 animals were controls.
The incidence of necrosis (partial and complete) is the same in both groups. ($\chi^2 = 0.26$. $P = 0.62$).
The distribution of the results of "no necrosis," "partial necrosis," and "complete necrosis," is essentially the same. ($\chi^2 = 0.46$ and $P = 0.95$).
The increase in the death rate of the treated animals is highly significant. ($\chi^2 = 16.6$ and $P = <0.0001$).

frostbite necrosis.

One hundred and ten animals were subjected to freezing at –15°C for 30 minutes; 72 of these were treated with 3 cc of heparin every 6 hours. The results are given in Table III.

The statistical evaluation of the results of the injury as determined by the chi-square test, including those cases in which the fate of the leg was determined at the time of death, shows that there is no significant difference between treated and untreated animals insofar as the incidence of necrosis is concerned ($\chi^2 = 0.256$ and $P = 0.62$). There is also no difference in the degree of final injury. If the results are computed with regard to the distribution of cases with "no necrosis," "partial necrosis," and "complete necrosis," $\chi^2 = 0.46$ and $P = 0.95$. On the other hand the death rate of the treated animals is significantly increased. ($\chi^2 = 16.6$ and $P = <0.0001$.)

*Fatalities During Heparin Treatment.* As already mentioned, the fatalities in the heparin-treated groups were much more numerous than in the corresponding controls. Of 153 heparin-treated animals 74 were lost during the 6 days of treatment whereas of 80 controls only 17 animals died. The difference in the death rate between these two groups is highly significant ($\chi^2 = 15.2$ and $P = 0.0001$). The fatalities in the heparin-treated series can be divided into two main groups. In one group, the animals exhibited a shock-like condition after the first or second injection of heparin without hemorrhages. The second group consisted of fatal hemorrhages which occurred in the course of the treatment.

It was soon apparent that fatalities without hemorrhages after the first injection of heparin were directly related to the time which elapsed between injury and heparin injection. This is shown for 72 cases under homogeneous experimental conditions and with intervals between injury and first injection of from 0-12 hours (Table IV). The increase in death rate occurring with increasing intervals between

TABLE IV.
Death Rate of Animals in Relation to the Time Interval Between Cold Injury and the First Injection of Heparin.

| Interval between injury and first injection | Survived | Died | Total |
|---|---|---|---|
| 0– 2 hr | 22 | 0 | 22 |
| 2– 4 " | 16 | 1 | 17 |
| 4– 8 " | 10 | 7 | 17 |
| 8–12 " | 8 | 8 | 16 |

The statistical evaluation shows that the increased death rate with increasing interval between injury and first heparin injection is significant. ($\chi^2 = 20.6$ and $P = 0.00015$).

injury and first injection is significant ($\chi^2 = 20.6$ and $P = 0.00015$).

The deaths from internal hemorrhages were far more numerous than those of the first type. Fifty-three of 153 heparin treated animals died from fatal hemorrhages into the peritoneal cavity, the retroperitoneal space, or into the lungs. The amount of heparin given before death occurred from hemorrhages differed greatly. Some animals developed hemorrhages after the first injection of 30 mg of heparin, others after more than 20 injections. On the average the animals which died from acute hemorrhages received 300 mg of heparin in a period of 60 hours by a regime of either 2 cc every 4 hours or 3 cc every 6 hours.

There was a distinct seasonal difference in the occurrence of hemorrhages during heparin treatment. Fatal hemorrhages occurred more often and with much smaller doses during the winter months. The reason for this observed seasonal difference is not apparent at this time.

*Discussion.* A comparison of our results with those of Lange *et al.* is possible only if the cold injury is determined by the physical conditions under which it occurs. We have shown in another investigation that under conditions which produce gangrene as a result of acute local cold injury, the immersed hind leg of a rabbit behaves practically like a dead body.[10] The time course of the temperatures in the exposed limb and the clinical results are very much the same in all animals investigated under the same standardized conditions. We should therefore be able to reproduce a comparable degree of cold injury by applying the same experimental conditions.

The cold injury in Lange's experiment was produced by two different procedures. One group of animals was exposed for 45 to 90 minutes to an alcohol dry ice bath which varied in temperature between –12°C and –20°C;[2] the other group was frozen for 30 minutes at –30°C.[5]

The data concerning the first group are not exact enough to permit reproduction. The extreme possibilities for these conditions include almost 100% probability for complete preservation of the immersed legs as well as 100% probability for complete loss. The second regime, *i.e.*, exposure for 30 minutes at –30°C, invariably produces very severe frostbite. But since Lange in a personal communication informed us that the experiments were conducted between –20°C and –30°C, and since in a control series at –25°C very severe frostbite always occurred, we used this latter temperature for the first part of our experiments. During this part of the experimental work the rubber boot was loosely attached by longitudinal strips, as described in the papers of Lange *et al.*[2] We failed completely to find any beneficial influence of heparin. Treated and untreated animals showed the same results. The number of animals was large enough to exclude the possibility that the results were due to random variation. (Table I.) We cannot give a reasonable explanation for the differences between Lange's and our results.

The maintenance of the blood coagulation time between 30 and 60 minutes is not possible when heparin is given intravenously at 12 or 6 hour intervals as described in Lange's publications. In 28 experiments we administered 2 cc of heparin intravenously to rabbits weighing 2,000-3,000 g. Blood clotting times were prolonged to 8 or more hours immediately, but within a few minutes they dropped sharply to reach the normal range within 4 hours or less. More exact investigations on the action of heparin will be published in another paper.

A more accurate evaluation of heparin in the prevention of frostbite gangrene was possible when we were able to perform the experiments with a degree of injury which did not preclude recovery. Systematic investigations showed that a feasible degree of injury could be obtained by an exposure to an alcohol bath of –15°C for 30 minutes with the protective rubber boot closely adherent to the depilated leg.

The relation of occurrence of frostbite to degree of injury is shown in Table V. The progression of the severity of injury from a bath temperature of –12°C to a bath temperature of –15°C with deep tissue temperatures of –3.3°C and –7.4°C, respectively, is rather

TABLE V.
Incidence of Necrosis in Relation to Degree of Injury.*

| °C for 30 min. | Temp. in deep tissue (No. of measurements in parentheses) °C | No necrosis | Partial necrosis | Complete necrosis | Total |
|---|---|---|---|---|---|
| 0 | +6.8 (2) | 12 | 0 | 0 | 12 |
| —5 | +2.2 (2) | 12 | 0 | 0 | 12 |
| —10 | —0.3 (7) | 25 | 1 | 0 | 26 |
| —12† | —3.3 (6) | 27 | 5 | 0 | 32 |
| —15 | —7.4 (14) | 4 | 16 | 12 | 32 |

* Animals which died before the fate of the tissue was decided are not considered.
† These animals were exposed to a bath temperature of —15°C with different conditions of conductivity (rubber boot not adherent). By measurement of the temperatures in the leg and by interpolation we determined this temperature to correspond to —12°C with the higher conductivity (rubber boot adherent).

striking. While at –12°C only 5 of 32 showed tissue necrosis, at –15°C 28 of 32 suffered gangrene. The distribution of the degree of injury in the group exposed to –15°C, *i.e.*, 12 cases of total gangrene, 16 cases of partial gangrene, and 4 cases without necrosis, should furnish an ideal basis for the evaluation of treatment of acute cold injury. The beneficial effect would become apparent in a shift to the groups of lesser injury.

As shown in Table III, the statistical computation of treated and untreated animals with this degree of injury does not show such a shift. There is significantly no difference in the distribution of the degree of injury between the two groups ($P = 0.95$).

Several other investigators who attempted to repeat Lange's experiments came to the same conclusions as we did. Quintanilla *et al.*[7] were not able to demonstrate any beneficial effect of heparin after various degrees of frostbite. Fuhrman and Crismon[6] in their experiments also did not obtain favorable results from heparin treatment.

It is difficult to explain the failure of several investigators to reproduce Lange's results. The only major point of Lange's experimental procedure which we did not follow was in the use of anesthesia. But Fuhrman and Crismon,[6] Schumacker *et al.*,[8] Quintanilla *et al.*,[7] anesthetized the animals before freezing and did not obtain better results.

The assumption that Lange *et al.* actually produced less severe injuries in their experiments than we did in reproducing them does not abolish this discrepancy. In the second phase of our experiments heparin proved to be ineffective even in the least degree of cold injury that is followed by gangrene. There remains the possibility that some of the factors not sufficiently controlled, such as species of animals used, food, acclimatization, temperatures at which the experiments were performed or at which the animals were kept during the time of observation, might prove to be of major importance.

The incidence of fatalities due to heparin treatment was of secondary interest in our investigations. However, it must be considered in the evaluation of heparin as a therapeutic agent. Furthermore, the death rate determines very much the value of the statistical analysis of the therapeutic results.

The statistical computation applied to our data is not correct in a strict sense. The results were tabulated under the headings of "no gangrene", "partial gangrene", "total gangrene", and "death". These groups are not comparable from a statistical point of view. We can reasonably assume that "no gangrene", "partial gangrene", and "total gangrene" constitute a sequence of increasing severity. Death of the animals either with or without treatment is not comparable to the other groups. Death occurred when, according to the clinical appearance, the injured leg was completely lost, or partially lost, or even completely saved. In 2 large groups we observed that the fatal outcome was due to the delayed first injection of heparin or to seasonal changes in sensitivity to heparin. We do not see how it is possible to determine whether the probability of dying or surviving under these conditions is or is not the same for the three

groups of final injury. In other words, our material underwent a change and we do not know whether this change was selective or not.

Furthermore, we cannot give a numerical representation for the groups "no gangrene", "partial gangrene", "total gangrene". Our experimental results do not enable us to assume that the extent of necrosis is a continuous function of increasing severity of injury since all types of tissue do not show the same sensitivity to cold.

The statistical data given in the paper serve only the purpose of showing that the constellation of figures on which our conclusions are based are not due to random variation.

*Summary.* A total of 153 rabbits had their legs exposed to temperatures of −25°C or −15°C for 30 minutes and subsequently treated with heparin administered intravenously for a period of 6 days. Twelve animals received 20 mg of heparin every 4 hours, 39 received 30 mg every 12 hours, and 102 received 30 mg every 6 hours.

Eighty animals were frostbitten at the same temperatures and for the same exposure times and used as untreated controls.

A statistical analysis of the results showed that tissue necrosis from frostbite was not significantly less in the heparin-treated animals.

Of 153 animals that were treated with heparin 74 died whereas 17 of 80 control animals succumbed during the experiment. The fatalities in the heparin-treated series were due to fatal internal hemorrhages in 53 cases; in 17 instances to a shock-like condition especially observed when the initial injection of heparin was delayed 4 or more hours after injury, and 4 animals died from unknown causes.

*Conclusions.* 1. The excellent results reported by Lange and his co-workers on the use of heparin in frostbite were not reproducible.

2. The results of heparin therapy by the regime recommended by Lange were not more satisfactory in our experiments than those in untreated control animals.

3. The death rate was significantly increased in animals that received heparin in doses proposed by Lange *et al.*

We wish to give credit to Mr. Allyn Kimball of the Department of Biometrics, School of Aviation Medicine, Randolph Field, Texas, for making the statistical analyses of the experimental data.

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## Alteration in Permeability of Some Membranes by Hyaluronidase and Inhibition of this Effect by Steroids. (17355)

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It is well established that hyaluronidase increases the "permeability" of the ground substance and can thereby markedly facilitate the spread of India ink, dyes, bacteria, drugs, etc. through the cutaneous tissues.[1,2] Hyaluronidase may also facilitate the penetration of some drugs into the cornea by attacking the monosulfuric ester of hyaluronic acid of this tissue.[3] It was of interest to investigate the effects of hyaluronidase on the permeability of membranes both *in vitro* and *in vivo* since this has not been done heretofore. Such a study is of timely interest in light of the possible relationship of hyaluronidase as a causative factor in arthritis and the alleviation of arthritis by steroids such as Compound E.[4,5]

Most of the *in vitro* tests were made using the urinary bladder of the rabbit as a semi-

[1] Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

[2] Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

[3] Seifter, J., *Ann. N. Y. Acad. Sci.*, in press.

permeable membrane. The *in vivo* studies were made by injecting a dye into the synovial cavity of rabbits and using the speed of appearance of dye in the urine and the rate of excretion into the urine as an index of permeability of the synovial membrane. These studies have been extended to human subjects under the influence of various drugs and diseases. In this paper we are presenting the data obtained *in vitro*. Those obtained *in vivo* correspond in nearly every detail to these and will be presented in a separate paper.

*Materials and methods.* The hyaluronidase* was prepared from bull testes and assayed 7000 to 9400 turbidity reducing units per mg of nitrogen. Membranes used in this study were the lens capsule, urinary bladder membrane processed according to the method of Barnes,[6] and freshly prepared urinary bladder of the rabbit. We also studied the effects of hyaluronidase on water imbibition by skeletal muscle, penetration of drugs into excised frog hearts, and filtration through isolated frog skin, because membrane permeability may be involved in these phenomena.

*Crystalline lens membrane.* Freshly excised, intact lens of frogs and rabbits were used. Series of test tubes were set up, four in each series, with one pair containing distilled water and the other pair physiological salt solution. One lens was placed in each solution, the tube agitated, and an aliquot of the supernatant removed. To one tube of each pair hyaluronidase was added to make a 0.05% solution, the tubes again agitated, and samples removed. The tubes were maintained constantly at 38°C. The degree of turbidity of each solution was read every five minutes, using the B scale of the Fischer Electrophotometer. A water clear solution gave a light transmission of 100%; an increase in turbidity decreased the amount of light transmitted (Fig. 1).

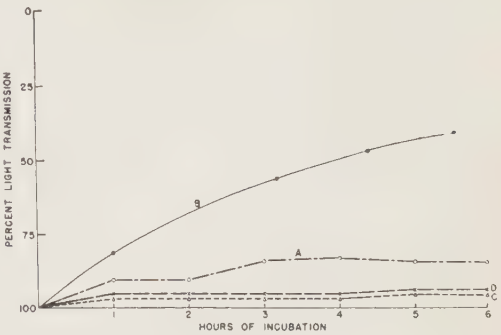


FIG. 1.

Turbidity of incubating medium containing whole rabbit lens (38°C). Fischer electrophotometer used for readings).

Curve A—Distilled water.

Curve B—0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in distilled water.

Curve C—Physiological salt sol.

Curve D—0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in physiological salt sol.

*Urinary bladder membrane.* The urinary bladder was removed surgically from rabbits by excision at the neck. Each membrane was prepared by washing and storing overnight at 36°C in 0.85% NaCl. The following morning a membrane was stretched and mounted over the open end of a thistle tube, the adventitia being toward the inside of the tube. After drying for 30 minutes in the hot air oven at 100°C, the membrane was waterproofed at the edge with paraffin and stored in the refrigerator until used. The preparation served as an osmometer with a semipermeable membrane separating M/1 sucrose on the inside from distilled water against the mucosal surface on the outside. One membrane could be used several times if after each experiment it were reprocessed by repeating the above procedure. When reprocessing was repeated on 5 separate membranes for 3 successive days the curves of the rate of osmosis obtained on the first day could be superimposed on those obtained on the third day, thus ruling out the possibility that reprocessing altered permeability. A total of 58 permeability experiments were done on 30 processed membranes.

Urinary bladder membranes maintained in the fresh state were also studied. After killing the rabbits by a sharp blow across the

4 Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 181.

5 Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 277.

* Hydase, Wyeth.

6 Barnes, T. C., Textbook of General Physiology, 1937, Blakiston Co., Philadelphia, Pa.

cervical vertebrae, the urinary bladder was immediately removed, washed in Tyrode's solution at 37 ± 1°C, and by means of a purse string suture of surgical silk secured as quickly as possible to the open end of a thistle tube. The adventitia was inside and the mucosa outside. Tyrode's solution containing M/1 sucrose was placed within the osmometer which was immersed in a beaker containing Tyrode's solution. Oxygen was bubbled into the solution on each side of the membrane. A freshly excised bladder was used for each of 21 experiments performed at 37°C in a hot air incubator.

The following were tested for their effect on permeability of urinary bladder membranes: (1) hyaluronidase; (2) adrenal cortical extract (10% alcoholic, Upjohn Co.); alcoholic solutions of cholesterol, testosterone, estrone, desoxycorticosterone acetate,† Equilenin,† and Equilin;† (3) alarm reaction induced by colchicine. The final concentration of alcohol in the steroid tests did not exceed 5 γ of ethanol per ml. This concentration was found to have no effect on membrane permeability. All substances tested for effect on permeability were added to the solution in contact with the mucosal surface.

*Skeletal muscle membrane.* The gastrocnemius muscle of frogs of near weight was immersed in a bath of 50 ml distilled water or 50 ml distilled water-drug solution, immediately removed from the solution and weighed after the water was shaken off. The muscle was then returned to its constant temperature bath maintained at 0 to 2°C. Thirty minutes after immersion and each one-half hour thereafter for 5 hours, the muscles were weighed. In several instances a weighing was also taken at 24 to 48 hours. Seventy-five muscles were used in this study and the following compounds were tested: hyaluronidase; an antihistaminic, Neohetramine; and histamine diphosphate.

*Results. Lens membrane.* Incubation of frog and rabbit lens in distilled water at 38°C resulted in progressive turbidity of the supernatant (Fig. 1, curve A). The lens capsule usually blistered in one hour and ruptured up to 6 hours later. Incubation in physiological salt solution, on the other hand, did not produce turbidity and did not injure the capsule (Fig. 1, curve C). Addition of 0.05% hyaluronidase produced a marked increase in the turbidity of the distilled water (Fig. 1, curve B) but had no effect on the appearance of the physiological salt solution (Fig. 1, curve D). Tests with anthrone reagent[7] revealed that the turbidity was not due to release of carbohydrate material. This reagent, however, does not detect hyaluronic acid or its breakdown products.

It could not be assumed from the turbidity that hyaluronidase affected the permeability

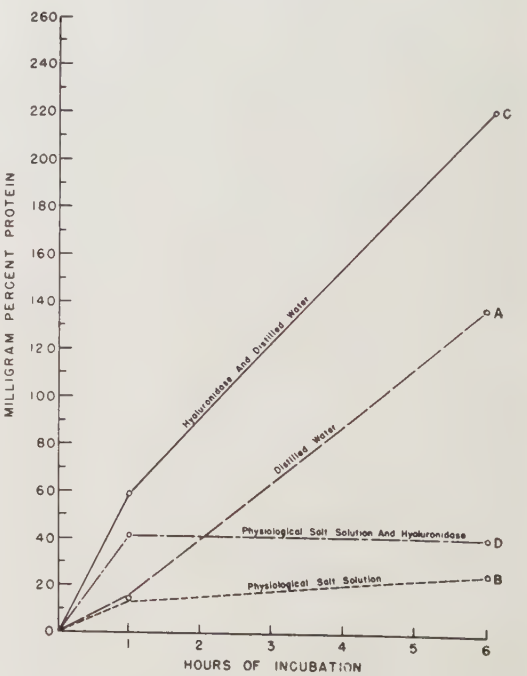


FIG. 2.

Leakage of proteins from rabbit lens exposed to hyaluronidase.

Curve A—Lens incubated at 37°C in distilled water. Capsule blistered in 1 hr.

Curve B—Lens incubated at 37°C in physiological salt sol.

Curve C—Lens incubated at 37°C in 0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in distilled water. Capsule blistered in 1 hr.

Curve D—Lens incubated at 37°C in 0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in physiological salt sol.

† We wish to thank the Schering Corp., Bloomfield, N. J., for the desoxycorticosterone acetate, and Ayerst, McKenna & Harrison Limited, Montreal, Canada, for the Equilenin and Equilin.

[7] Morris, D. L., *Science*, 1948, **107**, 254.

TABLE I.
Effect of Hyaluronidase and Steroids on the Permeability of Processed Membranes.

| | No. of exp. | Avg rise at 360 min. (mm) | Range (mm) | Median (mm) |
|---|---|---|---|---|
| Distilled water | 24 | 101 | 67-189 | 105 |
| Hyaluronidase | 10 | 245 | 130-360 | 240 |
| Adrenal cortical extract* | 3 | 91 | 62-124 | 87 |
| Adrenal cortical extract + hyaluronidase | 5 | 125 | 100-146 | 125 |
| Cholesterol* | 2 | 122.5 | 120-125 | |
| Cholesterol + hyaluronidase | 1 | 133 | | |
| Estrone* | 2 | 110.5 | 110-111 | |
| Estrone + hyaluronidase | 2 | 50 | 29- 71 | |
| Testosterone* | 1 | 93 | | |
| Testosterone + hyaluronidase | 1 | 176 | | |

* These membranes had much greater permeability in the control period when only distilled water was used.

only in distilled water, since the released material might be soluble in physiological salt solution. Protein determinations of the supernatants were made by the sulfosalicylic acid method, and some of the data are presented in Fig. 2. Each curve was obtained by averaging the milligrams percent of protein for the supernatants of 6 to 8 lenses. The data for distilled water and distilled water-hyaluronidase were taken only from lenses showing blistering or rupture in one hour in order to make a comparison with physiological salt solution-hyaluronidase which for some as yet unexplainable reason became inactivated in one hour. Leakage of protein through the membrane immersed in distilled water or physiological salt solution did not occur to a significant degree during the period when the lens was intact (curves A and B). Hyaluronidase caused leakage of protein through the membrane during this period (curves C and D). Individual capsules differed markedly in their resistance to injury by the hypotonicity of distilled water and to alteration of permeability by hyaluronidase.

*Urinary bladder membrane.* The results on the processed membranes are listed in Table I and typified in Fig. 3. The rate of osmosis through a semi-permeable bladder membrane is shown by Curve A. The distilled water at the end of the experiment was sugar free by the anthrone method. Two $\gamma$ of hyaluronidase per ml markedly increased the rate (Curve B). At the end of this experiment the concentration of sugar outside the membrane was 0.30 mg per ml as compared with 0.34 mg per ml inside; therefore the semi-permeable nature was abolished. The permeability effect was reversible since removing the hyaluronidase

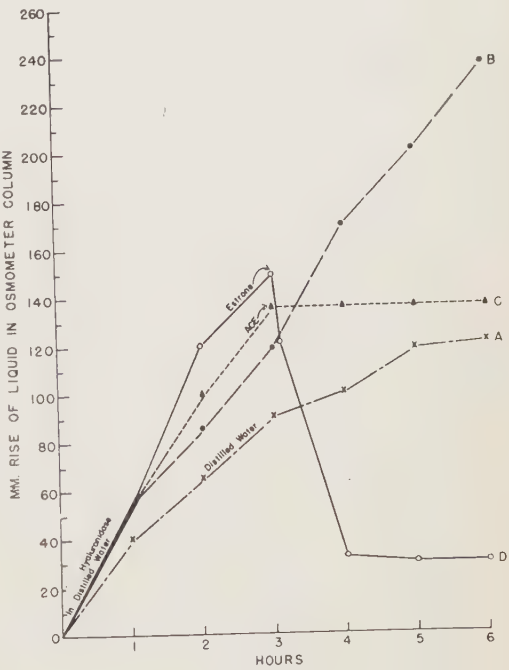


FIG. 3.

Rate of osmosis. (Urinary bladder membrane semi-permeable to M/1 sucrose).

Curve A—Distilled water outside osmometer.

Curve B—Hyaluronidase $1:10^6$ (assaying 7000 T.R.U. per mg N) in distilled water (2 $\gamma$ per ml).

Curve C—Same concentration of hyaluronidase in distilled water as in curve B, but aqueous adrenal cortical extract, 10 $\gamma$ per ml, added at the arrow.

Curve D—Same concentration of hyaluronidase in distilled water as in curve B, but aqueous estrone, U.S.P., 10 $\gamma$ per ml, added at the arrow.

TABLE II.
Effect of Hyaluronidase, Steroids, and an Alarming Stimulus on the Permeability of Surviving Membranes.

| | No. of expts. | Avg rise at 360 min. (mm) | Range (mm) | Median (mm) |
|---|---|---|---|---|
| Tyrode's sol. | 5 | 39 | 38- 40 | 39 |
| Hyaluronidase | 5 | 160 | 159-161 | 160 |
| Adrenal cortical extract + hyaluronidase | 2 | 74 | 74- 75 | |
| Estrone | 1 | 20 | | |
| Estrone + hyaluronidase | 1 | 13 | | |
| Desoxycorticosterone acetate | 1 | 104 | | |
| Desoxycorticosterone acetate + hyaluronidase | 1 | 150 | | |
| Equilenin + hyaluronidase | 1 | 3 | | |
| Equilin + hyaluronidase | 1 | 5 | | |
| Colchicine *in vivo* | 1 | 0 | | |
| Colchicine *in vivo* + hyaluronidase *in vitro* | 1 | 0 | | |
| Plasma from rabbit in alarm reaction | 1 | 13 | | |

after the first hour decreased the rate of osmosis, and adding it again at the third hour reestablished it. This alternation could be demonstrated for the first 6 hours.

Ten $\gamma$ of either adrenal cortical etxract, testosterone, or cholesterol per ml inhibited the passage of water through the membrane immersed in hyaluronidase solution. This observation resembles the *in vivo* inhibiting effect of adrenal cortical extract on the spreading activity of hyaluronidase[8] and decreased absorption through the synovial membrane.[9] Ten $\gamma$ of estrone per ml not only stopped osmosis through the hyaluronidase treated membrane, but reversed the flow (Curve D). Injection of estrone *in vivo* increased the resistance of the ground substance[10] and of synovial membranes[9] to hyaluronidase.

The results obtained with fresh membranes in oxygenated Tyrode's solution were identical with those just described and are listed in Table II. Further studies were carried out using an estrogenic type steroid. Twenty-four $\gamma$ of Equilenin or 30 $\gamma$ of Equilin per ml reversed the flow of water through the hyaluronidase treated membrane in a manner similar to that of estrone. When the compounds were removed and replaced with a fresh solution of hyaluronidase and Tyrode's solution, osmosis proceeded as it had before the steroids had been added indicating that the membranes were not injured.

Ten $\gamma$ of desoxycorticosterone acetate per ml enhanced considerably the effect of hyaluronidase on osmosis. To further study the action of this steroid, a rabbit was given 35 mg desoxycorticosterone acetate in oil intramuscularly once a week for two weeks. Os-


FIG. 4.

Influence of antihistaminics and histamine on lyophilia of frog gastrocnemius muscle (immersed in distilled water at 2°C).

Curve A—Distilled water.

Curve B—0.1% neohetramine hydrochloride in distilled water. (1% neohetramine hydrochloride causes the muscle to decrease below the original weight).

Curve C—0.1% to 0.2% histamine diphosphate in distilled water.

Curve D—0.1% neohetramine hydrochloride plus 0.1% to 0.2% histamine diphosphate in distilled water. (1% neohetramine counteracts 0.1% histamine diphosphate).

8 Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, in press.

9 Seifter, J., Baeder, D. H., and Begany, A. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, in press.

10 Sprunt, D. H., and McDearman, S., *Endocrinology*, 1940, **27**, 893.

mosis through this bladder was more rapid in Tyrode's solution alone than through normal bladder membranes treated with hyaluronidase.

In order to determine whether an alarm reaction with the concomitant release of adrenal steroids would affect the permeability of membranes, 3 mg colchicine per kg were administered subcutaneously to rabbits. Two such membranes were prepared. Osmosis was completely inhibited through them even in the presence of hyaluronidase.‡ One ml of plasma from a rabbit in colchicine alarm reaction immediately inhibited osmosis and reversed the flow through a normal membrane.

*Striated muscle membrane.* Thirty gastrocnemius muscles immersed in distilled water gained an average of 82% of their original weight in 5 hours, 87% in 24 hours, and 78% at 48 hours (Fig. 4, curve A). Neohetramine inhibited the uptake of water so that at 5 hours there was an average increase to 62%, at 24 hours 39%, and at 48 hours only 31% (typical curve, Fig. 4, curve B), thus confirming the observations of Halpern[11] that antihistaminic drugs decrease the permeability of skeletal muscle immersed in distilled water. The uptake of water in gastrocnemius muscles immersed in a solution containing 0.1 to 0.2% histamine diphosphate was 73% of the original muscle weight at 5 hours, 103% at 24 hours, indicating a slight "edema" effect, and 98% at 48 hours (typical curve, Fig. 4, curve C). This concentration of histamine was sufficient to counteract the "anti-edema" effect of the lower concentrations of the anti-histaminic compound (Fig. 4, curve D). Neohetramine in a concentration of 1%, however, antagonized the effect of 0.1% histamine diphosphate.

Addition of hyaluronidase in amounts up to 1% in the distilled water solution alone or in the distilled water containing histamine or Neohetramine did not result in deviations from the usual behavior of the compound tested. The curves obtained in each instance could be superimposed on those shown in Fig. 4. Similar results were obtained with the rabbit soleus muscle incubated at 37°C.

The experiments with skeletal muscle are illustrative of our finding that hyaluronidase does not enhance penetration of drugs in isolated frog hearts and does not affect ultrafiltration through frog skin.

*Summary.* 1. Purified testicular hyaluronidase had no effect on the normal or altered permeability of skeletal muscle, cardiac muscle, or of isolated frog skin.

2. Hyaluronidase strikingly increased the permeability of the lens capsule and urinary bladder membranes, and abolished their semipermeable character.

3. The enhancing effect of hyaluronidase on osmosis through the bladder membrane was increased by desoxycorticosterone acetate.

4. Adrenal cortical extract, testosterone, and cholesterol abolished the enhancing effect of hyaluronidase. Estrone, Equilin, and Equilenin reversed the direction of flow through hyaluronidase treated membranes.

5. Hyaluronidase did not alter the complete inhibition of osmosis through the bladder membrane of rabbits which had been exposed to an alarming stimulus.

6. The products released *in vivo* during the alarm reaction rendered bladder membranes impermeable even in the presence of hyaluronidase.

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[11] Halpern, B., personal communication. Published reference unavailable.

‡ Preliminary *in vitro* and *in vivo* experiments indicate that cortisone acetate is as effective as the alarm reaction.

# Effect of Sulfomucopolysaccharides on Growth of Tumor Tissue.* (17356)

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Fischer[1] found that injury regeneration in tissue cultures is partially or completely suppressed by heparin. By treating transplants of Flexner-Jobling rat carcinoma with heparin, Goerner[2] could suppress its growth. Zakerzewski[3–6] made a systematic investigation of the influence of heparin on the growth of different tissues *in vitro*. He found that the growth of normal embryonic tissue was suppressed by heparin and that the same thing was true with Jensen sarcoma. He assumes that the growth-suppressing effect of heparin depends on an antiprothrombin action. Zakerzewski has also investigated the growth-suppressing effect of heparin on Jensen sarcoma in rats and on polymorphocellular sarcoma in mice. He could show that less tumor growth and less necrosis resulted from intravenous treatment. Fischer,[7] using pure heparin preparations, later showed that the suppressing effect of heparin on blood-coagulation and tissue growth disappeared after boiling with hydrochloric acid for a short time.

Balazs[8] has shown that other sulfonated mucopolysaccharides (the sodium salt of agar acid and the calcium salt of chondroitinsulfuric acid) as well as heparin suppress the growth of embryonic tissue *in vitro*. This suppressing effect depends on negative groups that are part of the polysaccharide. He could also show that there was a parallelism between the growth-suppressing effect and the number of negative groups per molecule. Only the undifferentiated tissues were found to be sensitive to the substances in question.

It is not only the growth of proliferated tissues from higher animal species that is suppressed by heparin. Fischer and Nyström[9] showed that the growth of yeast cells was also suppressed by heparin. Heilbrunn and Wilson[10] were recently able to demonstrate that heparin suppressed the mitosis of Chaetopterus eggs after fertilization. This action is reversible and has no degenerating effect on the eggs. They assume that heparin prevents the mitotic gelation.

*Experimental. In vitro.* Our *in vitro* experiments were performed on tumor cells from methyl cholanthrene tumors of the rat. All of the experiments were performed on cultures in Carrel flasks using a 2 phase system. (D 5). The cultures were placed in chick plasma without embryonic extract. In all the experiments the fluid phase of the control cultures has consisted of embryonic extract and physiological saline. Instead of the physiological saline, equivalent amounts of sodium heparinate (0.4 mg per flask) were added to the tissue cultures in one series, and an equivalent amount of the sodium salt of agar acid in another series. In preparing the sodium heparinate we used the powdered form having 13.35% moisture: S, 11.47% and N, 2.00%. This preparation we have gratefully received from Professor E. Jorpes. The sodium salt of agar acid preparation was made according to Hoffman and Gortner from the commercial agar. The preparations were dissolved in physiological saline solution (Ringer), and the pH was controlled between 6.7 and 7.2. In both cases the substances in question were added to cultures 24 hours old.

* This work was aided by the Wenner-Gren Foundation, Sweden.

1 Fischer, A., *Virchows Arch.*, 1930, **94**, 279.

2 Goerner, A., *J. Lab. Clin. Med.*, 1930, **16**, 369.

3 Zakerzewski, Z., *Z. f. Krebsforsch.*, 1932, **36**, 513.

4 Zakerzewski, Z., *Bull. Internat. de l'Acad. Pol. Sci. et Lettre Cl. Med.*, 1932, 238.

5 Zakerzewski, Z., *Arch. f. exp. Zellforsch.*, 1932, **13**, 152.

6 Zakerzewski, Z., *Klin. Woch.*, 1932, **11**, 113 and 158.

7 Fischer, A., *Protoplasma*, 1936, **26**, 344.

8 Balazs, A., in press.

9 Fischer, A., and Nyström, P., *Biochem. Z.*, 1933, **262**, 364.

10 Heilbrunn, L. V., and Wilson, W. L., Proc. Soc. Exp. Biol. and Med., 1949, **69**, 179.

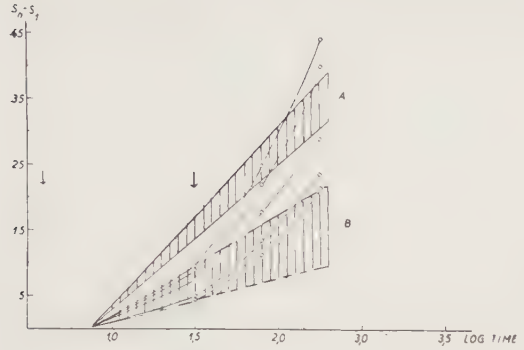


FIG. 1.

Growth of the methyl cholanthrene tumor of rats in a Carrel culture flask. Growth index: $S_n-S_1$. $S_n$ is the measured growth at different times and $S_1$ is the growth of the first day (before addition of the fluid phase). The shaded area A represents the control culture (embryonic extract plus Ringer's solution in fluid phase). The shaded area B shows the cultures where the liquid phase has been added twice (embryonic extract plus heparin 25 mg%). The solid lines show the cultures where the fluid phase has been added once (embryonic extract plus heparin 25 mg%). The time of growth is given on a logarithmic scale. Arrows show the changes of the fluid phases.

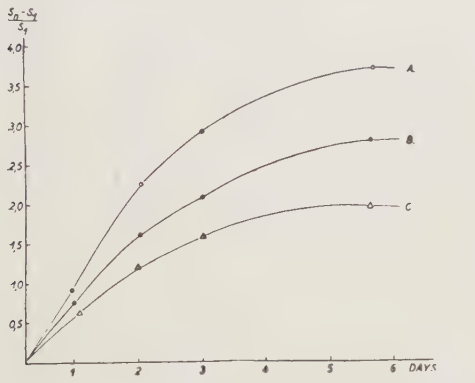


FIG. 2.

The growth of the methyl cholanthrene tumor of rats in Carrel culture flasks. Growth index: $\frac{S_n-S_1}{S_1}$ ($S_n$ is the measured growth at different times, and $S_1$ is the growth of the first day).

A. Embryonic extract plus Ringer's sol.

B. Embryonic extract plus sodium heparinate (40 mg%).

C. Embryonic extract plus sodium salt of agar acid (40 mg%).

Before the fluid phase was added, the entire culture with the growth area was measured ($S_1$). The growth area of the cultures ($S_n$) were then determined every day. Data can be obtained from Fig. 1 and 2.

Fig. 1 illustrates the growth of those cultures which were treated with sodium heparinate (0.2 mg per flask). In the experiments, controls and experimental cultures are included. The fluid phase always contains the same concentration of growth promoting, water soluble, thermolabil substances of embryonic (12 days old chicken embryos) tissue-extract. To this fluid medium was added the test substances. The time is given logarithmically and indicates that the growth of the control cultures can be represented by a straight line from which the growth of the experimental cultures deviates markedly. When heparin is added once to the fluid phase an obvious suppression of the growth is observed; but this suppression is later compensated. This compensation shows itself by a marked increase in growth, in some cases even greater than that of the controls.

The heparin fluid phase in another series of cultures was changed twice. Under these conditions the growth was uniformly suppressed during the entire course of the experiment as shown by Fig. 1. Similar to the control cultures, the cultures repeatedly treated with heparin show a linear growth.

In an additional experiment, the growth of the cultures during continuous addition of sodium heparinate (40 mg % in every flask) and the sodium salt of agar acid was studied. Fig. 2 is a graphic representation of the relative growth of the cultures. In this case, the time scale is not logarithmical. It can be seen from the figure that the growth of the experimental cultures is considerably less than that of the controls. The most marked suppression of the growth was obtained when the agar salt was used, but the sodium heparinate also gives a similar suppression of growth. Using standard methods we have not been able to observe any difference in the morphology of the cells in the control and the cells in the experimental cultures.

*In vivo.* As our experiments have shown that the growth of tumor cells is suppressed by sodium heparinate and the sodium salt of agar acid, it seemed interesting to us to investigate whether the cellular growth could also be suppressed *in vivo*. Attempting to answer this question we have inoculated intraperi-

toneally suspensions of cells from a solid Ehrlich's carcinoma into 300 white male mice (20-25 g) in 3 experiments.

In the first experiment the animals were given sodium heparinate together with the tumor cells intraperitoneally. In another series the animals were treated intraperitoneally with sodium heparinate for a few days after the tumor had been inoculated. In both these experiments it was found that the experimental animals started to die a few days later than the control animals. However, this difference was not persistent and the mortality curves gradually coincide. In a third experiment we have given a more consistent treatment. One hundred fifty mice were divided into 3 groups of 50 each. All were inoculated with an equal amount of tumor cell suspension (1.5 million cells in 0.1 ml physiological saline). At the same time 50 animals were given 0.3 mg sodium heparinate and 50 animals 0.3 mg of the sodium salt of agar acid subcutaneously. The third group served as a control. The experimental animals were treated daily with the same quantity of sodium heparinate and sodium salt of agar acid. The results obtained are seen from Fig. 3. The mortality of each of the series is calculated. It can be seen from the figure that the lines which represent the mortality of the 3 groups run parallel, which suggests that the difference between the groups is constant during the time of the experiment. At the end of the experiment, 11 days after the inoculation, 82% of the control animals, 72% of the heparinate treated animals, and 66% of the animals treated with agar extract had died. The experiment, it seems to us, indicates that there is a real difference in mortality between the control and experimental animals. As the groups are relatively large this difference could hardly be due to mere chance but is by all probability due to the treatment.

*Discussion.* Our results seem to confirm completely the statement of Zakerzewski that heparin suppresses the growth of tumor tissues *in vitro*. In addition to that we have shown that the sodium salt of agar acid has the same effect. The question arises how the effect of these sulfomucopolysaccharides is

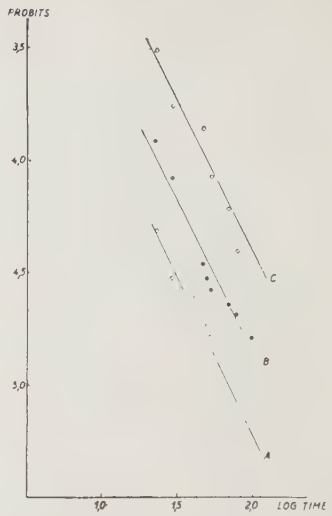


FIG. 3.

Dosage-mortality curve of ascites tumor of mice. Probits units are plotted against the logarithm of the time.

A. Control animals.

B. A total of 3 mg sodium heparinate injected subcutaneously during 9 days.

C. A total of 3 mg of the sodium salt of the agar acid injected subcutaneously during 9 days.

All the animals in the three groups have been given 1.5 million Ehrlich's carcinoma ascites cells intraperitoneally. The dosage-mortality curve has been calculated according to Bliss, C. L., *Quart. J. Pharmacol.*, 1938, **11**, 192.

brought about *in vitro*. From this point of view there are two possibilities: either the sulfomucopolysaccharides used have a neutralizing effect on the growth promoting substance present in the embryonic extract and the original tissue, or they act directly on the growing cell. Whether or not the sulfomucopolysaccharides in the latter case suppress mitosis and migration, we cannot decide. The effect seems to be specific as shown by Balazs.[8] He found that the heart tissue from chick embryos pulsates faster and longer in a substrate containing sodium heparinate than in the control cultures containing embryonic extract. Using a suitable concentration of sodium heparinate, the growth could be completely suppressed in the former case.

Concerning the experiments *in vivo* it seems clear to us that death was delayed by treatment with sodium heparinate and the sodium salt of agar acid. We have not been able to prevent the death of the animals; but one must consider the fact that we are dealing

with a form of tumor which under these experimental conditions in 12 to 15 days produces a 100% mortality in mice. How the treatment has influenced the tumor cells is, of course, hard to tell. Three possibilities seem acceptable:

1. The sulfomucopolysaccharides used have a neutralizing effect on the toxic products derived from the tumor cells.

2. The sulfomucopolysaccharides exercise a general resistance-increasing effect on the tissues of the organism.

3. The sulfomucopolysaccharides in question exercise a direct growth-suppressing effect on the tumor cells.

Naturally, we have not been able to decide how the substances in question act; we only wanted to suggest these possibilities. It is of interest to note that Cramer and Simpson[11] and Holmgren and Wohlfart[12] have shown that in methyl cholanthrene tumors in mice and rats, great masses of mast cells can appear. Cramer and Simpson point out that the mast cells in the skin increase in number before the tumors are developed, and that resistant mice show an ample number of mast cells in the skin. They further point out that the reaction of the mast cell is involved in a defensive process directed against the development of skin cancer. They also point out that this is of interest as the mast cells contain heparin (Holmgren and Wilander[13]), Jorpes, Holmgren, and Wilander.[14] It is also of interest that hyaluronic acid and other mucopolysaccharides are widely distributed in different tissues. Balazs and Holmgren[15] have shown that sulfomucopolysaccharides appear in injured tissue where they with all probability have a suppressing effect on the growth of the regenerating tissue.

*Summary.* Sulfomucopolysaccharides (sodium heparinate and the sodium salt of agar acid) suppress the growth of cells from methyl cholanthrene tumors in the rat *in vitro*. To ascertain whether the substances in question had a similar effect on growing tissues *in vivo* mice were inoculated intraperitoneally with the suspension of cells from Ehrlich's carcinoma, after which they were treated in various ways with sodium heparinate and with sodium salt of agar acid. It was found that the mortality of the control animals was greater than that of the treated animals. The results and various possible explanations are discussed.

[11] Cramer, W., and Simpson, W. L., *Cancer Res.*, 1944, **4**, 601.

[12] Holmgren, Hj., and Wohlfart, G., *Cancer Res.*, 1947, **7**, 686.

[13] Holmgren, Hj. and Wilander, O., *Ztschr. f. mikr. anat. Forsch.*, 1937, **42**, 242.

[14] Jorpes, E., Holmgren, Hj., and Wilander, O., *Ztschr. f. mikr. anat. Forsch.*, 1937, **42**, 279.

[15] Balazs, A., and Holmgren, Hj., *Cell. Res.*, 1949, in press.

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## Detection of Mumps Virus in Mice Sacrificed at Different Periods of Time after Injection. (17357)

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During the past 2 years we have experimented with the cultivation of mumps virus in embryonated chicken eggs, and have prepared inactivated virus vaccine for laboratory use and trial in human subjects.[1]

In the course of a portion of this work, we have recently attempted to infect (looking for both apparent and inapparent infections) several of the smaller laboratory animals. To date, we have not been able to bring about any such infections or produce definite gross or microscopic lesions. We have used "raw" periembryonic egg fluid virus, and also such virus after concentration by the alcohol

[1] Muntz, H. M., Powell, H. M., and Culbertson, C. G., *J. Lab. and Clin. Med.*, 1949, **34**, 199.

TABLE I.

Complement Fixation Tests of Periembryonic Fluids from Eggs Injected with Mouse Tissue Dilutions for Assay of Mumps Virus. Mice sacrificed 1 hour after injection of virus.

| Mouse No. | Route inj. | Mouse organ | Dilution: 1 part in | Complement fixation titer of egg fluids 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ip | Brain | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 2 | ip | | 10 | ++ | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 3 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 4 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 1 | ip | Liver | 10 | ++++ | ++++ | ++++ | ++ | + | — |
| | | | 100 | ++++ | ++++ | — | — | — | — |
| 2 | ip | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 3 | in | | 10 | +++ | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 4 | in | | 10 | ++ | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 1 | ip | Spleen | 10 | ++++ | ++++ | ++++ | ++ | — | — |
| | | | 100 | ++++ | ++ | — | — | — | — |
| 2 | ip | | 10 | ++++ | ++++ | ++++ | ++++ | + | — |
| | | | 100 | ++++ | +++ | + | — | — | — |
| 3 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 4 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 1 | ip | Lung | 10 | ++++ | ++++ | +++ | ± | — | — |
| | | | 100 | ++++ | ++++ | ++++ | ++ | — | — |
| 2 | ip | | 10 | ++++ | ++++ | ++++ | + | — | — |
| | | | 100 | ++++ | + | — | — | — | — |
| 3 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 4 | in | | 10 | not tested | | | | | |
| | | | 100 | +++ | + | — | — | — | — |
| 1 | ip | Testes | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 2 | ip | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 3 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |
| 4 | in | | 10 | — | — | — | — | — | — |
| | | | 100 | — | — | — | — | — | — |

Legend: ip indicates intraperitoneally; in indicates intranasally.

method. Our negative results are in conformity with those of others who used mainly "raw" (*i.e.* non concentrated) virus. In the course of these tests, however, we have determined the titers of mumps virus in various organs and tissues at different times after injection of active virus, and the results of such tests showing appearance and disappearance of mumps virus in mice seem of sufficient interest to report.

Two groups of Swiss mice were injected with Enders strain[2] of mumps virus in periembryonic fluid. This virus had a 4+ complement fixing (C.F.) titer of 1:16.[3] One group of mice received doses of 1.0 cc of this virus intraperitoneally. The other group of mice received doses of 0.05 cc of this virus intranasally. Pairs of mice from each group were

[2] Virus and information obtained from Dr. J. F. Enders, Boston, Mass.

[3] Bengtson, I. A., *Public Health Rep.*, 1944, **59**, 402.

sacrificed at 1 hour, 24, 48, 72 and 96 hours and at 2 and 3 weeks after injection. Dilutions of the homogenized organs obtained at autopsy were injected into 7 day embryonated eggs, and after proper incubation the periembryonic fluids from these eggs were tested for the presence of mumps virus by the C.F. test.

The results of the first test of mouse organs and tissues removed one hour after injection of mumps virus are shown in Table I. It is observed that spleen, lung and liver contained considerable virus. A trace of virus was found in the brain, while none was found in the testes. Although further negative results are not tabulated here, we found the virus neither in the pancreas, heart muscle, nor blood.

In these experiments, the mice which received intranasal virus got only one-twentieth the amount of virus given intraperitoneally. This intranasal dose however was sufficient to give rise to demonstrable titers of virus in the liver and lung one hour after injection.

Twenty-four hours after injection of mumps virus into mice we found, at autopsy, only traces of virus in the spleen, lung, liver and brain. As before, no virus was found in the testes, pancreas, heart muscle, and blood.

No mumps virus was found in the organs and tissues heretofore examined in mice sacrificed at 2, 3, and 4 days, and at 2 and 3 weeks after injection of virus. Negative results after these longer periods of time indicate that inapparent infection does not occur.

*Summary.* Mumps virus administered to mice intraperitoneally or intranasally appears within an hour in the lungs, liver, spleen, and to a lesser extent in the brain (in the latter case after intraperitoneal injection only.) Sojourn of viable virus in organs where found is short. Testes, pancreas, heart muscle, and blood were found not to contain virus at any time. Mice used in these tests of mumps virus did not exhibit symptoms of toxicity at all comparable to mice injected at different times in the past with influenza virus, although these 2 viruses are somewhat similar otherwise.

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## Production of Acute Experimental Circulatory Failure by Graded Pulmonary Artery Constriction.* (17358)

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Because of the practical and technical limitations of studying congestive heart failure in human subjects, numerous attempts have been made to produce heart failure in animals employing many different methods. Various drugs have been administered, including chloroform, ethyl alcohol, potassium chloride, chloral hydrate, and diphtheria toxin,[1] posterior pituitary extracts,[2] and quinidine,[3] in an effort to depress the myocardium. In addition, irritants have been injected into the pericardium[4] and myocardium;[5] the ventricular wall has been severely cauterized,[6] the coronary arteries ligated,[7,8] and the myocardial capillaries plugged with foreign sub-

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‡ Sarah Welt Fellow.

1 Fahr, G., and Buehler, M. S., *Am. Heart J.*, 1943, **25**, 211.

2 Nolasco, J. B., and Kohrman, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 108.

3 Reiss, R. A., and DiPalma, J. R., *Am. J. Physiol.*, 1948, **155**, 336.

4 Armstrong, T. G., *Quart. J. Exp. Physiol.*, 1940, **30**, 263.

5 Luisada, A., *Medicine*, 1940, **19**, 475.

6 Starr, I., Jeffers, W. A., and Meade, R. H., *Am. Heart J.*, 1943, **26**, 291.

7 Orias, O., *Am. J. Physiol.*, 1932, **100**, 629.

8 Gross, L., Mendlowitz, M., and Schauer, G., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 446.

stances.[9] In general, there have been two serious drawbacks to these methods: 1) the difficulty in controlling the severity of the experimental procedures, and 2) concomitant undesirable side effects, such as induction of arrhythmias and toxic actions on other organs of the body.

Since a reduced cardiac output—variously occasioned—is probably the initiating factor leading to the typical signs of congestive heart failure, the prerequisite for any technic attempting to produce experimental circulatory failure is a reduction of the output of the heart without lowering the blood pressure to shock levels. Therefore, we have employed a graded pulmonary artery stenosis as an experimental approach to the problem. This procedure possesses the following virtues: the cardiac output may be reduced significantly without concurrent marked changes in arterial pressure; a strain is placed on the right ventricle; any desired gradation of severity may be applied rapidly and easily; and finally, the procedure is readily reversible.

Previous workers who successfully produced experimental pulmonic stenosis were interested in its relation to pulmonary embolism[10] or in the cardiac dynamics of such a lesion,[11] and only incidental attention was directed to the effects on the peripheral circulation. Furthermore, there is only a narrow range of constriction which will give changes simulating clinical heart failure. If the constriction is too mild, right atrial and aortic pressures remain unaltered, and the only observable result is some increase in the right intraventricular pressure during systole. On the other hand, if the degree of constriction exceeds the optimum, an uncompensated drop in cardiac output occurs with a decrease in arterial blood pressure to shock levels.

Since we plan to utilize this technique in the future to determine the primary effects of reduction of cardiac output upon renal function, it seemed advisable to supplement our knowledge of the dynamic cardiovascular changes which are produced by increasing the resistance to right ventricular ejection. In addition, an attempt was made to elucidate the mechanism by which the central venous pressure becomes elevated during this procedure.

*Methods.* Mongrel dogs weighing between 8 and 16 kg were anesthetized with morphine followed by intravenous injection of sodium barbital (ca. 200 mg/kg). The chest was opened through the midline and respiration was maintained artificially. The pulmonary artery was dissected free near its origin, and was constricted by means of a strong cord noose attached to a screw device, permitting fine gradations of circular constriction. In each of the 13 experiments which were successfully performed, right atrial and aortic pressures were optically registered by means of modified Gregg manometers. In 5 of these experiments, right intraventricular pressures were also recorded, and in 5 others the pressures in the pulmonary artery distal to the point of constriction were recorded. A calibration was made at the end of each record in relation to a fixed base-line.

*Results.* No alterations in right atrial or aortic pressures could be detected until the pulmonary artery was constricted to a certain critical degree, estimated by previous workers[10] to be about 60% of occlusion. Pressure pulses registered from the aorta and right atrium during progressive, gradual constriction of the pulmonary artery are presented in Fig. 1 and 2. For reasons outlined by Wiggers,[12] right atrial pressures were measured at 3 points in all curves:

1. A point—the maximum point attained during atrial systole;

2. Z point—just prior to closure of the A-V valves; and

3. V point—just prior to opening of the A-V valves. These three points are indicated on the right atrial curve of record A in Fig. 1.

Using these criteria, records B and C of

[9] Roos, A., and Smith, J. R., *Am. J. Physiol.*, 1948, **153**, 558.

[10] Gibbon, J. H., Jr., Hopkinson, M., and Churchill, E. D., *J. Clin. Invest.*, 1932, **11**, 543.

[11] Fineberg, M. H., and Wiggers, C. J., *Am. Heart J.*, 1936, **11**, 255.

[12] Wiggers, C. J., *Physiology in Health and Disease*, 5th edition, Lea & Febiger, Philadelphia, 1949.

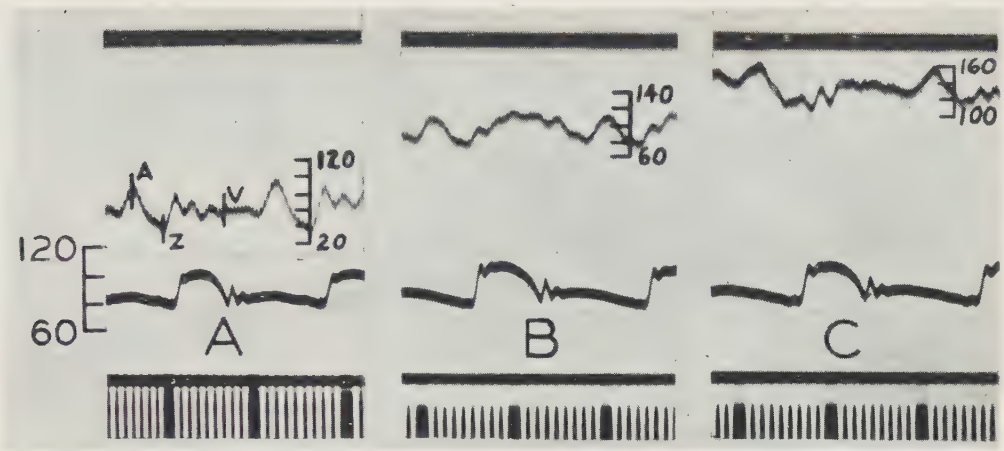


FIG. 1.

Right atrial (upper) and aortic (lower) pressure curves in experimental pulmonic stenosis. Record A—control; record B—minimal constriction beyond the critical degree; record C—slight additional constriction.

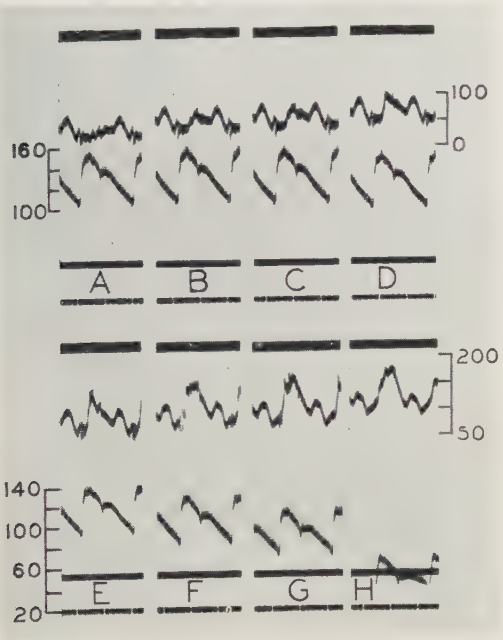


FIG. 2.

Right atrial (upper) and aortic (lower) pressure curves during control (record A) and progressively more severe pulmonic stenosis (records B through H). Discussion in text.

Fig. 1 reveal that a two-stage mild constriction of the pulmonary artery caused a relatively large elevation of the right atrial pressure as compared to control record A, with no significant alteration in the aortic pressure. This rise in right atrial pressure is present at all points measured, but is most pronounced at the V point, amounting to an increment of 46 mm water in B, and of 83 mm water in C. The aortic systolic pressure remained within 5 mm Hg and the diastolic within 1 mm Hg of the control levels, the larger pulse pressure being due to a slight slowing of the heart. Such records show that lesions causing increased resistance to right ventricular discharge can effect an increase in central venous pressure without alteration of the arterial pressure and without graphic evidence that left ventricular discharge has been decreased.

We next studied an entire series of progressively severe, graded constrictions of the pulmonary artery in order to ascertain whether immediately recognizable criteria could be established which enabled one to determine that a significant reduction in cardiac output had been produced without lowering the arterial pressure to shock levels. The effects of such a sequence of graded constrictions are presented in Fig. 2.

Minimal constriction of the pulmonary artery beyond the critical point has elicited a 35 mm water elevation of right atrial pressure at the V point in record B and a further increase to 40 mm water in C, as compared to control record A, with no significant alteration in the aortic pressure. The first significant decrease in the aortic pressure is

present in record D, falling to 149/102 mm Hg, as compared to the control value of 155/110 mm Hg in record A. This slight drop in aortic pressure is accompanied by an additional rise in right atrial pressure of 55 mm water above control. This then is the first definite indication that the output of the left ventricle has been diminished.

With further compression of the pulmonary artery, the changes in right atrial and aortic pressures become more pronounced, as observed in records E through H. Even in record G, the aortic pressure is still within normal limits (116/80 mm Hg), while the central venous pressure has risen to 123 mm water above control levels, measured at the V point. Very slight additional constriction then produced a fall in aortic pressure to shock levels (record H). Throughout the entire series of records, no significant change in heart rate is present.

The circulatory conditions represented in records D to G simulate the cardinal features of clinical congestive heart failure, namely elevation of central venous pressure, reduction of cardiac output, and an arterial pressure within normal limits. We have been able to maintain these conditions for four hours or more, requiring only minimal adjustments of the clamp. Therefore, we shall attempt to reproduce these stages during our anticipated studies on the effects of reduced cardiac output upon renal function. The conditions existing in record H would, of course, be unsatisfactory since the arterial pressure is within shock levels. Stages B and C also would be unsatisfactory, since minute changes in cardiac output which might be present would probably be within the limits of error of any method for measuring the cardiac output (*e.g.* Fick principle).

A more detailed perusal of Fig. 2 reveals additional information which is not directly pertinent, but which is of some importance. It is evident in the right atrial curves registered during relatively severe stenosis of the pulmonary artery (records E through H) that a steep rise in slope occurs during ventricular systole, reaching a peak near the end of ventricular systole. These curves simulate closely those described by Little during experimental tricuspid regurgitation,[13] and were recorded at a time when the central venous pressure was very high and the right ventricle was markedly dilated. Preliminary tests utilizing the injection of a small amount of concentrated dye (T1824) into the right ventricle tended to confirm the presence of tricuspid insufficiency. Within 3 to 5 seconds after injection during severe pulmonic stenosis, the concentration of dye in the right auricle and right ventricle were almost identical, and the amount of dye recovered from both sites was very large.

To further supplement our knowledge of the altered circulatory dynamics of pulmonic stenosis, pressures were recorded in several experiments from the pulmonary artery distal to the point of constriction during progressive compression of the pulmonary artery. As illustrated in Fig. 3, slight constriction beyond the critical point elicited a vibratory irregularity representative of a systolic murmur, as seen in the pulmonary artery curves of records B and C. In record B, the aortic pressure was slightly depressed, although quite frequently a pulmonic murmur was observed before any alteration occurred in the aortic or right atrial pressures. Furthermore, in some experiments the pulmonary artery and aortic pressures were slightly elevated when constriction just sufficient to produce a murmur was applied. In the pulmonary artery curves of records C and D, a sharp peak is present at the very beginning of systole. This is analogous to the anacrotic incisura described by Katz, Ralli, and Cheer in experimental aortic stenosis.[14] More severe constriction eventually resulted in a marked fall of systolic, diastolic, and pulse pressures in the pulmonary artery, as seen in record D. With release of constriction, these pressure changes were reversed, often with a transitory elevation of pulmonary artery pressures above control levels.

Right intraventricular pressures were re-

[13] Little, R. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 602.

[14] Katz, L. N., Ralli, E. P., and Cheer, S., *J. Clin. Invest.*, 1928, **5**, 205.

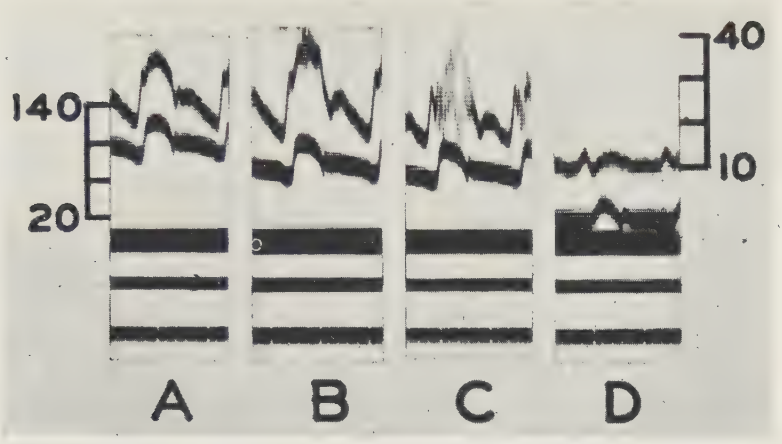


FIG. 3.
Pulmonary artery (upper) and aortic (lower) pressure curves during the control period (record A) and during gradually progressive pulmonic stenosis (records B through D).

corded in several experiments during experimental pulmonic stenosis, and the findings were in accord with those reported by Fineberg and Wiggers.[11] To repeat these findings briefly, degrees of occlusion below about 60% elevated the systolic but not the diastolic pressures (initial tensions), while aortic and right atrial pressures were unaffected. This systolic pressure rise is believed to be a passive phenomenon, due solely to the increase in resistance, with no alteration in the force of ventricular contraction. Further degrees of occlusion eventually produced an elevation of initial tension as well as of systolic pressure. However, very soon after the rise in initial tension appeared, the systolic pressure began to diminish as the constriction progressed. This was considered by Fineberg and Wiggers to be evidence of myocardial failure, due to tremendous stretching of the myocardium, perhaps combined with diminished coronary flow, the result of decreased aortic pressure.

*Discussion.* As previously reported by Gibbon, Hopkinson, and Churchill,[10] progressive compression of the pulmonary artery beyond about 60% of occlusion produces a gradual elevation of central venous pressures and a fall in aortic pressures. With carefully graded constriction, we were usually able to reach a point where a moderate elevation of right atrial pressure could be elicited with no significant alteration in the aortic pressure. With more severe degrees of occlusion, aortic pressures dropped, the right ventricle became markedly dilated, and curves were often obtained which were characteristic of tricuspid regurgitation. In attempting to explain the elevation of central venous pressures during this procedure, the marked venous distention observed during severe pulmonic stenosis indicates that the principal factor is an increase in the quantity of blood within the central veins. It is frequently stressed by

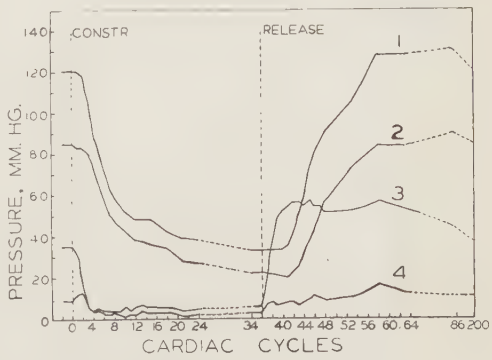


FIG. 4.
Plot showing the effects in successive beats of an abrupt occlusion and subsequent release of the main pulmonary artery on aortic systolic (1) and diastolic (2) and on pulmonary systolic (3) and diastolic (4) pressures, the latter two recorded distal to the occluding clamp. Discussion in text.

present-day investigators that it is dynamically impossible to produce an elevation of the venous pressure combined with a reduction in the cardiac output unless the blood volume is augmented. To prove this concept, many involved and complicated experimental procedures and physical models have been contrived.[6,15,16] Simple and decisive expedients, such as were used in this research, have largely been ignored. Our results demonstrate unequivocally that an increase in total blood volume is not an obligatory factor in causing an elevation of the central venous pressure, since extreme pressure variations can be produced within a few seconds, and can be maintained for many hours. The increase in the volume of blood contained within the central veins during our experiments is independent of any changes in total blood volume, and must be due to redistribution of the blood already present within the circulatory system.

Sufficient reduction of the lumen of the pulmonary artery acts primarily to diminish the output of the right ventricle. Initially, therefore, following constriction of the pulmonary artery there is a brief period in which the venous return to the heart exceeds the amount of blood pumped away by the right ventricle, resulting in a progressive increase in central venous pressure. Due to the decreased cardiac output, however, the venous return gradually diminishes, until it again becomes equal to the reduced cardiac output. The right atrial pressure then becomes restabilized, but at a higher level of pressure. It is within the relatively brief period during which there is an inequality between the cardiac output and venous return that a redistribution in the circulating blood volume has occurred. The problem resolves itself into an attempt to determine the source of the blood which contributes to the venous return over and above the suddenly diminished output of the right ventricle during this short period.

Fig. 4 presents graphic proof that a shift of blood does occur between the pulmonic and systemic circulations during pulmonic stenosis. Following a sudden drastic occlusion of the pulmonary artery, the systolic and diastolic pressures in the pulmonary artery distal to the point of constriction dropped abruptly, and the pulse pressure was reduced almost immediately to minute values. However, systolic, diastolic, and pulse pressures in the aorta diminished very gradually over the course of about 30 beats. Thus, while the entry of blood into the pulmonary circuit was markedly reduced, blood still was extracted from the pulmonary vascular bed at almost the normal rate for a few cardiac cycles, and at a slowly diminishing rate for many more. When the constriction was suddenly released, the pulmonary arterial pressures rose almost immediately to super-normal values, while the aortic pressures recovered much more gradually, indicating a refilling of the depleted pulmonary vessels to normal. Thus, our data favor the concept that the lungs serve an auxiliary function as a rather efficient blood reservoir interposed between the right and left ventricles. In addition to a shift of blood from the pulmonic to the systemic circuit, it is very possible that a transfer of blood occurs within the systemic circuit itself. This may take place from the arteries, capillaries, and small veins toward the central veins, or blood might be released from the various blood reservoirs located on the systemic side of the circulation, notably the spleen in the dog. This possibility deserves further study.

*Summary and conclusions.* An experimental attempt was made to simulate acutely the cardinal circulatory changes of congestive heart failure for the purpose of creating a preparation in which the incipient effects on renal function could later be studied.

Optically recorded pressure pulses from the aorta, pulmonary artery, and right atrium are presented, and show that judicious compression of the pulmonary artery can reduce the output of the left ventricle significantly without incurring a drastic fall of arterial pressure. These dynamic studies also revealed that such graded pulmonary artery constriction results in (a) a reduction of pulmonary sys-

[15] Starr, I., and Rawson, A. J., *Am. J. Med. Sc.*, 1940, **199**, 27.

[16] Starr, I., *Am. J. Med. Sc.*, 1940, **199**, 40.

tolic and diastolic pressures distal to the constriction, and the development of a systolic murmur, (b) a rise in maximal and initial tensions in the right ventricle, and (c) an elevation of right atrial pressures. In some instances, relative tricuspid insufficiency developed.

The elevation of central venous pressure is obviously associated with translocation of a large volume of blood to the venous side of the circulation. Evidence is presented that a part of this transfer occurs from the pulmonary vascular bed, although it is admitted that a shift may also occur within the systemic circuit itself toward the central veins.

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## Cultivation of Poliomyelitis Virus in Cultures of Human Foreskin and Embryonic Tissues.* (17359)

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Recently, the propagation *in vitro* of the Lansing strain of poliomyelitis virus in human embryonic tissues was reported and evidence presented that this virus is capable of multiplying in cells other than those of nervous origin.[1] These experiments have been continued and this agent now has been carried for a total period of 224 days through 13 serial cultures in which the tissue consisted of mixed human embryonic skin and muscle. This strain has also been maintained for 173 days in two lines of 11 serial cultures each and composed respectively of human embryonic intestine and brain.

Additional experiments described here in a preliminary manner are reported. Two objectives were in mind: (a) to determine whether the Lansing strain was capable of multiplying in completely differentiated non-nervous tissue as well as in embryonic tissue; (b) to determine whether the Brunhilde strain of poliomyelitis virus—a strain immunologically distinct from the Lansing group[2] and not adaptable to rodents—could, like the Lansing strain, be cultivated in non-nervous human embryonic tissues.

*Propagation of the Lansing strain in human foreskin tissue.* As a source of completely differentiated non-nervous tissue fragments of human foreskin were employed. The use of this tissue was suggested by the report of Blank, Coriell, and Scott,[3] who explanted it on the chorioallantoic membrane according to the method of Goodpasture. The material was derived from patients between 4 and 11 years of age. Each prepuce was sufficient for the preparation of at least 8 cultures and before mincing was washed 2 or 3 times in nutrient fluid[4] containing 50 units each of streptomycin and penicillin per ml. The fluid phase of the cultures, which contained the same concentration of antibiotics was removed and replaced at intervals of 4 days. The original set of cultures was inoculated with 0.1 cc of a 10% suspension of pooled mouse brains infected with the Lansing strain. As inoculum for subcultures 0.1 ml of the pooled

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† Work done while a post doctorate Fellow of the United States Public Health Service.

‡ Senior Fellow in Virus Diseases of the National Research Council.

1 Enders, J. F., Weller, T. H., and Robbins, F. C., *Science*, 1949, **109**, 85.

2 Bodian, D., Morgan, I. M., and Howe, H. A., *Am. J. Hyg.*, 1949, **49**, 234.

3 Blank, H., Coriell, L. L., and Scott, T. F. McNair, PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 341.

4 Weller, T. H., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 124.

TABLE I.
Multiplication of Lansing Poliomyelitis Virus in Human Foreskin Tissue.

| Culture set | No. of nutrient fluid changes prior to subculture | Day of incubation subculture done | Mouse $LD_{50}$ of pooled fluids used to inoculate subcultures | Calculated dilution of original inoculum at time of subculture |
|---|---|---|---|---|
| Original* | 4 | 20 | $10^{-1.0}$ | $10^{-6}$ |
| 1st subculture | 3 | 16 | $10^{-1.7}$ | $10^{-11}$ |
| 2nd " | 3 | 16 | $10^{-1.2}$ | $10^{-16}$ |
| 3rd " | 4 | 20 | $10^{-0.0}$ | $10^{-22}$ |
| 4th " | 3 | 16 | $10^{-1.6}$ | $10^{-27}$ |
| 5th " | (in progress) | | | |

* The $LD_{50}$ of the infected mouse brain used in the original inoculum was $10^{-3.7}$; 0.1 cc of a 1:50 dilution of brain was employed and the calculated mouse $LD_{50}$ titer of the fluid phase immediately after inoculation was equal to $10^{-0.5}$.

centrifuged fluids removed on the 16th or 20th day of cultivation from the preceding set of cultures was used.

In the experiment summarized in Table I the virus was maintained for 88 days through 5 serial cultures of human foreskin. During this interval the fluid phase was removed and replaced 17 times. Pooled centrifuged supernatant fluids removed from the 4th culture series on the 20th day of cultivation when inoculated intracerebrally produced paralysis in a rhesus monkey. Histological changes consistent with a diagnosis of poliomyelitis were observed in the monkey's cord. Supernatant fluids removed from each serial culture produced paralysis in mice on intracerebral inoculation. Mice injected with fluids from uninoculated control cultures of tissues from the same sources remained well. It was calculated that during the 88-day period of cultivation the suspension of virus originally inoculated had undergone a dilution of at least $10^{-27}$. It would appear, therefore, that the Lansing strain is capable of multiplication in the presence of well differentiated skin and subcutaneous tissue and in the absence of intact nerve cells.

*Propagation of the Brunhilde strain in human embryonic tissues.* Two series of experiments were initiated utilizing the same technic. In one, the cultures consisted of mixed skin, muscle and connective tissue from the extremities of human embryos of 3 to 4 months gestation, and in the other of brain tissue from the same embryos. In each, the original set of cultures was inoculated with 0.1 cc of a 10% suspension of monkey cord infected with the Brunhilde strain of poliomyelitis virus, supplied through the kindness of Dr. H. A. Howe. The skin and muscle series was subcultured three times to fresh tissues and the brain series twice.

Following intracerebral inoculation into rhesus monkeys of pooled centrifuged supernatant fluids of the skin-muscle series on the 12th and 21st days, respectively, from the second and third subcultures, muscular weakness of the lower extremities was observed. Histologic examination of the cords from these animals revealed changes consistent with infection by the virus of poliomyelitis. Fluids from the second subculture of the series prepared with brain tissue also produced paralysis on intracerebral inoculation into a rhesus monkey. No illness resulted after intracerebral inoculation of a portion of the same pooled supernatant fluids, from the 3rd subculture into Swiss white mice.

These findings indicate that the Brunhilde strain had been maintained for 73 days in the skin-muscle cultures and 39 days in those of brain. It was calculated that during the longer period the original inoculum of virus had been diluted $10^{-22}$ times as a result of subculture and changes in the fluid phase.

According to Bodian[5] the usual titer of monkey cord infected with this strain for the rhesus monkey is $10^{-6}$. From the calculated dilution of the primary inoculum of the tissue cultures, it may, therefore, be inferred that this strain, like the Lansing virus, also multiplies *in vitro* in the presence of non-nervous tissue. Whether or not it can be propagated

[5] Bodian, D., *Am. J. Hyg.*, 1949, **49**, 200.

indefinitely under these conditions must await further investigation.

We are grateful to members of the Staff of the Boston Lying-in Hospital and the Children's Hospital for supplying the tissues. We also acknowledge with thanks the indispensable assistance in the experimental work of Marguerite Buckingham and Jeanette Levens.

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## Fluorocardiography (Electrokymography) During Normal Respiration. (17360)

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Previous studies[1,2] described an apparatus, utilizing our modification of the Henny-Boone method of electrokymography,[3,4] and the typical patterns of tracings recorded over the major cardiovascular and pulmonary structures. Fluorocardiography was suggested as more descriptive than previously employed names for the method.

In previous studies, the tracings were recorded during voluntary apnea in an intermediate phase of respiration. Comparative observations showed that the pulsations of the lung (and, to a lesser extent, those of the hilar shadows and the pulmonary artery) were greater during inspiration than in sustained expiration. For practical purposes, an intermediate phase was chosen; a few words of instruction usually sufficed to teach the patient to hold his breath without excessive inflation or deflation of the lungs. This procedure was followed without demonstrable disadvantage in our entire series of clinical cases during 1947 and 1948. On the other hand, it was found difficult to obtain reliable tracings in children and patients with chronic diseases of the lungs who were unable to control their respiration because of age or dyspnea. For this reason, an attempt was made to overcome this difficulty by technical means.

*Technical aspects.* The problem was presented to Maurice B. Rappaport, E.E. He suggested that a filter may be introduced to minimize respiratory effects though with the introduction of some error.

Wandering of the base line caused by respiration may be reduced considerably by altering the electrical time constant of the apparatus. When this constant is shortened, there is a proportionally greater attenuation of the coarse as compared with the more rapid waves. Respiration is represented by extremely slow waves in the fluorocardiogram, far coarser than any of those caused by cardiac action. A suitable condenser, or filter, introduced between the fluorocardiograph and the recording galvanometer, may modify the time constant of the apparatus; the smaller the condenser, the lesser is the electrical constant. Condensers of various size, which may be introduced by means of a switch, permit selection of the optimum time constant, namely the minimum value producing a fluorocardiogram that does not wander off the paper. The reduction of the electrical time constant of the apparatus does introduce a certain degree of error in the fluorocardiogram which is dependent upon the amount of reduction. The error presents itself in two forms: one is a slight error in the phase (or actual time) of registration of the component waves; the other is a slight change in their configuration. However, there is no elimination of the essential component waves present

1 Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 336.

2 Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 348.

3 Henny, G. C., and Boone, B. R., *Am. J. Roentgen.*, 1945, **54**, 217.

4 Henny, G. C., Boone, B. R., and Chamberlain, W. E., *Am. J. Roentgen.*, 1947, **57**, 409.

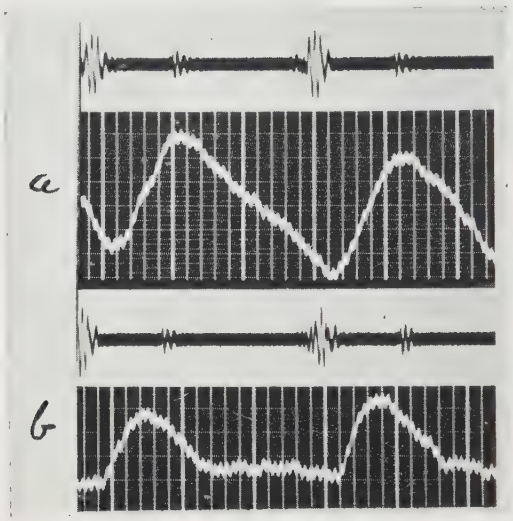


FIG. 1.
Pulsations of the pulmonary parenchyma (visible base of right lung) in a normal subject in apnea.
(a) Conventional tracing (densogram).
(b) Tracing obtained by adding an electrical filter.

under normal operating conditions. The filter used was arranged with an adjustable degree of low frequency damping. For optimal results, the degree of filtration was selected in each case to give a readable tracing with a minimal distortion of the fluorocardiogram.

Using this filter, it was possible to record the pulsations of the pulmonary vessels or any other cardiovascular structure during normal respiration with the following precautions:

(a) Whenever possible, the subject is instructed to breathe evenly and slowly, without sudden gasps.

(b) The roentgenologist observes the structure which is being studied and makes sure that it is not moving in and out of the slit during the respiratory movements.

In the practice, that degree of damping is chosen which will decrease the slow respiratory swing of the beam to a minimum with only slight diminution in amplitude of the waves caused by cardiac action. As shown in Fig. 1, a slight deformation of the waves is unavoidable. However, comparative studies on the height and initial rise of the pulse, made with the same degree of damping under otherwise similar conditions, proved the observations reliable. By means of the filter, several new studies were made possible.

*Results.* (A) *Fluorocardiogram of the child.* This fluorocardiogram, obtained by means of the above described filter, still presents occasional irregularities or wandering of the base line. This is due to the fact that children frequently exhibit a sharp beginning and end of inspiration with an abrupt change in phase; the filter cannot prevent slight wandering of the base line because of the irregularity and frequency of the respiratory waves. Nevertheless, clearly visible cardiac waves were obtained over all those cardiovascular and pulmonary structures which had been studied previously only in adults (Fig. 2). Now the field is open for the study of congenital heart diseases in childhood. In particular, the observation of a reduced amplitude of the pulsations of the pulmonary parenchyma is promising in the diagnosis of pulmonary stenosis.

(B) *Emphysema and pulmonary fibrosis with or without cor pulmonale.* In previous observations, we studied patients with chronic cor pulmonale who had only mild dyspnea and were able to hold the breath during the recording of fluorocardiograms. Other subjects with severe dyspnea yielded only poor tracings. By using the above described filter, two cases of chronic cor pulmonale with severe dyspnea were studied. As shown by Fig. 3, the details of the pulsations of the pulmonary and aortic knobs, as well as the pulsations of the lungs were recorded without difficulty.

(C) *Congestive failure.* Several cases of congestive failure have been studied by using this filter. These cases, which previously yielded only poor tracings, are currently studied and interesting observations are made concerning the lesser circulation.

(D) *Elimination of slow waves of cardiac origin.* In certain cases, a combination of cardiac disorders in an individual hinders a careful study of each. One patient observed by us had tricuspid insufficiency and auricular flutter. The study of the right auricle by means of the conventional apparatus revealed a systolic plateau similar to that previously described by us in the left auricle of patients

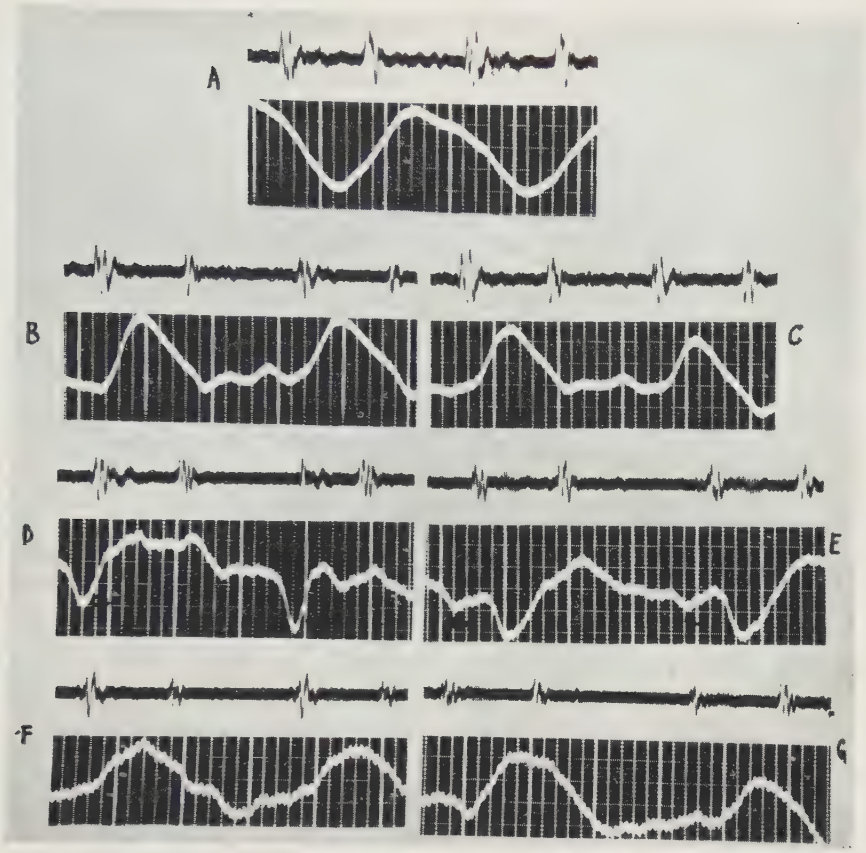


FIG. 2.

Tracings recorded on a 5-year-old normal boy during normal respiration with the use of the electrical filter.

A — Left ventricle; B = Pulmonary arch; C = Aortic arch; D = left auricle (right oblique); E = Right auricle; F = Right hilum; G = right lung; a—auricular wave of the auricular tracings.

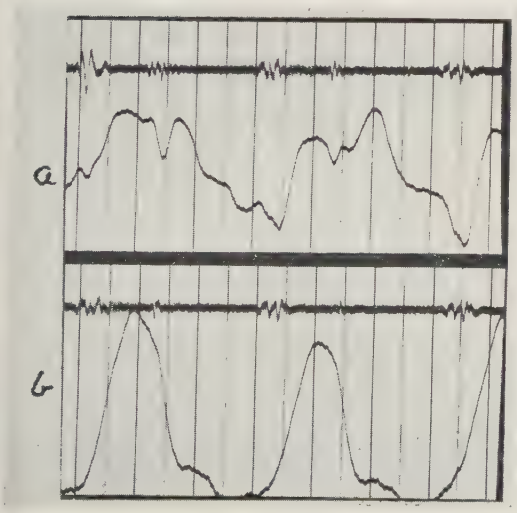


FIG. 3.

Tracings obtained during respiration by the use of the filter in a patient with severe pulmonary emphysema and dyspnea (border tracings). (a) Pulmonary knob; (b) Aortic knob.

with mitral insufficiency and due to regurgitation of blood from the respective ventricle.[5] On the other hand, use of the filter permitted recognition of the rapid auricular rate of contraction (300 per minute) by eliminating part of the slow wave caused by the tricuspid insufficiency (Fig. 4).

*Summary.* (a) Fluorocardiography during normal respiration is possible by the use of an additional electrical filter, the physical

[5] Luisada, A. A., and Fleischner, F. G., *Am. J. Med.*, 1948, **4**, 791.

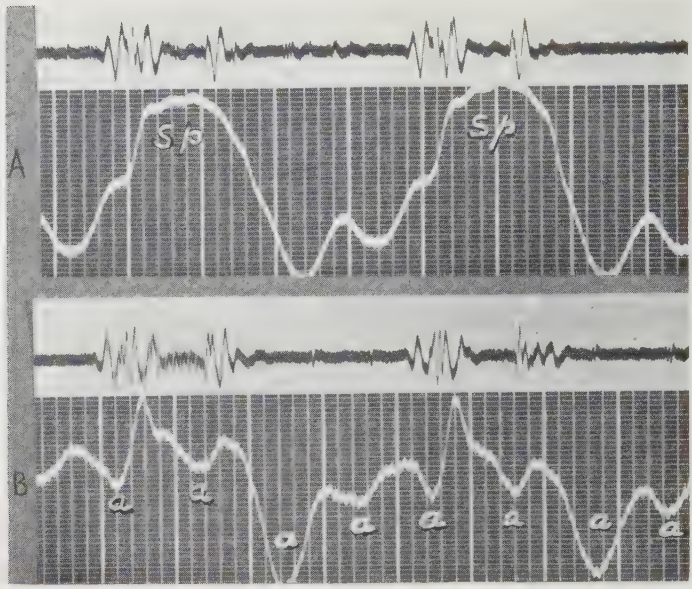


FIG. 4.

Tracings obtained during respiration in a patient with congestive failure, tricuspid insufficiency, and auricular flutter.

(A) Border tracing of right auricle (conventional tracing). A systolic plateau (SP) due to valvular insufficiency is apparent.

(B) Border tracing of right auricle (use of the filter). The rapid succession of auricular contractions (rate of 300) becomes apparent (a,a,a).

basis and principle of which are given.

(b) By using the filter, it is possible to record the fluorocardiogram of the child. This is of particular importance in congenital heart diseases.

(c) By using the filter, it is possible to study dyspneic cases of emphysema, pulmonary fibrosis, or congestive failure.

(d) The use of the filter eliminates those slow waves of cardiac origin which may interfere with other rapid cardiac waves whose study is more important in particular cases.

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## Determination of Iodine-131 in Urine.* (17361)

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In conjunction with research on the diagnostic and therapeutic value of radioactive iodine (8.0 day I-131) in thyroid disorders, a simple and accurate procedure for determining the amount of radioiodine excreted is needed. Preparation of counting samples by evaporation of urine samples to dryness is a tedious and unpleasant procedure, and has also proved inaccurate, perhaps due partly to loss of some iodine by volatilization (possibly as volatile organic iodine compounds) and partly to variations in the geometrical character of the resulting solid samples.

* The iodine 131 used in this investigation was supplied and obtained on allocation from the Atomic Energy Commission.

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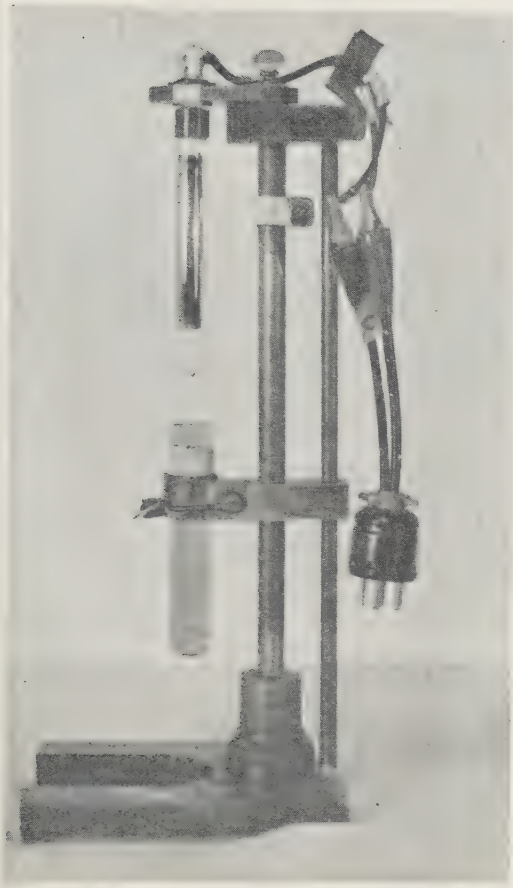

FIG. 1.
Shows the dipper counter (top) and sample tube (bottom) on microscope base.

A procedure for direct counting of the I-131 radiations in the urine by means of a dipping counter has been devised along lines similar to those reported by Solomon and Estes[1] for aqueous solution measurements. A thin-walled glass dipping counter (Radiation Counter Laboratories, Mark 1, Model 80) was mounted on a modified microscope base, as shown in Fig. 1. Samples of urine (sometimes diluted with water) were placed in test tubes whose length was exactly that of the glass portion of the counter tube (about 11 cm), and whose inside diameter was about 2 cm, 5 or 6 mm greater than the outside diameter of the counter tube. In the experiments reported here, 8.5 ml of liquid proved optimum for covering the sensitive area of the counter when counter and sample tube were arranged concentrically.

*Experimental.* To reduce errors due to variations in geometry, all samples in a given series, including the standard urine, were counted in the same sample tube. Since the counter tubes showed some photosensitivity, the external surfaces of the sample tubes were blackened with India ink to exclude light.

The counter tube was used in conjunction with either a 64 scaler (Nuclear Instrument and Chemical Corp., Model 165) or a decimal scaler (Berkeley Scientific Company, Model 1000).

*Background Count.* Backgrounds were determined over a fifteen minute period with the sample tube containing 8.5 ml distilled water arranged in the experimental position.

*Standard Urine Count.* Standard urine samples were prepared by addition of a known volume of a previously standardized aqueous solution of I-131 to a known volume of normal urine. Samples of 8.5 ml of the resulting spiked urine were counted with the dipping counter, yielding values expressed as counts per minute per microcurie of I-131 per milliliter of urine. About ten microcuries per liter of urine makes a satisfactory standard (about 1000 counts per minute.)

*Experimental Samples.* Urine of the patients was collected in vessels containing 1 ml of KI solution (20 mg/ml), to decrease loss of iodine by volatilization. The specimens were then sampled (8.5 ml) for direct counting, or if too active (over 8000 counts per minute for 8.5 ml sample), were diluted with water to yield satisfactory counting samples. Counting periods were adjusted so as to yield a total of 5000 to 10,000 counts, so that standard errors were less than 2%. In addition, samples were counted in triplicate (3 separate 8.5 ml samples, placed successively in the same sample tube) to reduce errors due to variations in geometry and sampling. After each sample was counted, the sample tube and counter tube were washed twice with water, once with 6 N $HNO_3$, and rinsed 3 times with distilled water. It was found advisable to

[1] Solomon, A. K., and Estes, H. D., *Rev. Sci. Instru.*, 1948, **19**, 47.

check the background for a brief period after each count to determine whether decontamination was complete.

*Effect of Dilution of Urine.* Samples of a standard urine giving 195 counts per minute per 8.5 ml were diluted with varying amounts of water, and 8.5 ml samples of the dilutions were counted with the experimental apparatus to determine whether the diluted urine exhibited the same counting efficiency as the undiluted. Samples of total volume 8.5 ml were prepared, containing the following amounts of the standard urine: 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 ml.

*Results and Discussion.* Results of the experiment to determine effect of diluting the radioiodine-containing urine are shown in Fig. 2. It is apparent from the figure that addition of water to the urine does not affect the counting efficiency of the radiations, in spite of the variation in specific gravity. This is the anticipated result if the counts obtained are due largely to the gamma radiations from the I-131, as expected. The beta particles from this nuclide (0.60 Mev) should be essentially completely absorbed by a water layer of about 2 mm thickness.

Comparison of the experimental sample


FIG. 2.
Effect of dilution of urine.

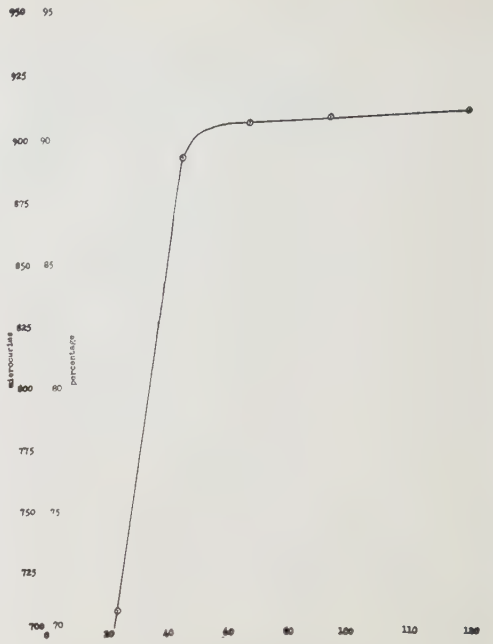


FIG. 3.
Urinary output of $I^{131}$. Tracer dose 100 microcuries.

counts (applying the dilution factor, if any) with the standard urine counts yields values in microcuries of I-131 per milliliter of experimental urine. Total output for the period over which the urine was collected is then obtained simply by multiplying this value by the total urine volume. Failure to collect the urine quantitatively is of course the greatest source of error in the measurements. Correction for radioactive decay since dosage may be applied either from the theoretical decay curve, or by counting the standard urine at the time of the experimental counts. Fig. 3 shows an actual series on a patient given 1000 microcuries of I-131. The curve indicates urinary output in microcuries and percentage, at daily intervals after administration.

Experiments now in progress are directed towards determination of the value of the urine measurements (*i.e.* rate of excretion of I-131) in diagnosis of thyroid disorders. It is believed that the determination of I-131 in urine is considerably more accurate than direct counting of the thyroid glands, which may vary considerably in size and shape, leading

to serious variations in geometry.

*Summary.* A method for the determination of Iodine-131 in urine is described. A dipping counter mounted on a modified microscope base using a blackened test tube with a sample large enough to cover the sensitive area of the tube was used. Counting of gamma radiations is quite reproducible by this procedure.

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## Effect of Procaine Hydrochloride on Response of the Heart to Epinephrine During Cyclopropane Anaesthesia. (17362)

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While studying the production of ventricular fibrillation in dogs by the intravenous administration of epinephrine during cyclopropane anaesthesia, observations were made on the effect of procaine HCl on the cardiac irregularities. Its effect on the rate and rhythm of the heart during cyclopropane anaesthesia and its effect on the cardiac response to epinephrine were noted.

*Method.* A modification of Meek's technic was used.[1] Thirty minutes after premedication with 1.0 mg/kg morphine sulphate and 0.04 mg/kg atropine sulphate, 18 male and female dogs (4-10 kg in weight) were induced with a cyclopropane and oxygen mixture, intubated, and allowed a further 30 minutes to equilibrate with the cyclopropane-oxygen mixture administered by a to-and-fro carbon dioxide absorber. The mixture was adjusted to produce stage III, plane 3 anaesthesia. A total of 78 injections of epinephrine 0.01-0.03 mg/kg were made, taking 20 seconds for each injection, and with at least 20 minutes between injections. Procaine HCl 5-15 mg/kg in 5 cc saline was given in 50 seconds, 5 minutes before 32 of the epinephrine injections. On 7 occasions 50 mg/kg of procaine HCl were given and not followed by epinephrine. Electrocardiograms were recorded continuously during and following each injection. When ventricular fibrillation occurred, artificial respiration with 100% oxygen was instituted, thoracotomy performed, and cardiac resuscitation attempted using manual massage and intracordial injections of procaine HCl 5-15 mg/kg.

*Effect on Cardiac Rate and Rhythm.* The cardiac rate was increased in all but two experiments when procaine HCl was given. (Table I). The degree of acceleration present 5 minutes after the injection increased with the dose employed (Table II). On 24 occasions irregularities were present at the time procaine HCl was injected, and with the exception of one sinus arrhythmia these changed to a regular sinus rhythm. (Table I).

*Modification of Cardiac Response to Epinephrine.* The response of the heart to epinephrine was notably different after the administration of 5-15 mg/kg of procaine HCl. Epinephrine was given 46 times without previous procaine HCl and 32 times following this agent. The occurrence of various rhythms following the epinephrine is shown in Table III. Calculation of "p" value[2] reveals a significant increase in the number of normal rhythms, a significant decrease in the number of cases with a sinus arrhythmia component in the rhythm, and no significant change in the incidence of either extrasystoles or of ventricular tachycardia.

An attempt was made to determine whether increased vagal tone was contributing to the electrocardiographic picture obtained following epinephrine injections. Signs taken to mean increased vagal tone were initial slowing, A-V block, and suppression of the sinus

[1] Meek, W. J., Hathaway, H. R., Orth, O. S., *J. Pharm. Exp. Therapy*, 1937, **61**, 240.

[2] Mainland, D., *Can. J. Research*, 1948, E, **26**, 1.

TABLE I.
Effects of Intravenous Procaine HCl (5-15 mg/kg) on Cardiac Rate and Rhythm Under Cyclopropane.

| Rhythm before procaine HCl | No. of exp. | After procaine HCl | |
|---|---|---|---|
| | | Reg. sinus rhythm | Acceleration |
| Regular sinus rhythm | 8 | 8 | 7 |
| Sinus arrhythmia | 18 | 17 | 18 |
| Heart block | 2 | 2 | 2 |
| Bigeminal rhythm | 3 | 3 | 3 |
| Irregular bradycardia | 1 | 1 | 0 |
| | 32 | 31 | 30 |

TABLE II.
Cardiac Acceleration with Procaine HCl.

| Dose, mg/kg | No. of exp. | Avg rate when procaine given | Avg rate 5 min. after procaine |
|---|---|---|---|
| 5 | 12 | 73.7 | 93.0 |
| 15 | 20 | 73.2 | 119.0 |
| 50 | 7 | 62.4 | 138.5 |

TABLE III.
Rhythms Produced by Intravenous Epinephrine.

| | No. of injects. | Rhythms observed | | | |
|---|---|---|---|---|---|
| | | Reg. sinus | Vent. tachy. | Ex. systol | Sinus arr. |
| Epinephrine alone | 46 | 2 | 17 | 17 | 9 |
| Epinephrine after procaine HCl | 32 | 9 | 10 | 10 | 0 |
| "p" values | | $<0.01$ | $>0.7$ | $>0.7$ | $<0.05$ |

node with a slow nodal or ventricular rhythm. Evidence for an increase in vagal tone was absent in only ten of 46 records where epinephrine was given alone, but was absent in 29 of 32 records where epinephrine followed procaine HCl. The p value for this difference is $<0.001$.

Ventricular fibrillation occurred in 2 of 17 animals when epinephrine was administered alone, and in 3 of 12 when epinephrine was preceded by 5 mg/kg of procaine HCl. These 3 animals had all received the same dose of epinephrine in previous experiments with the onset of ventricular extrasystoles or ventricular tachycardia only. We were unable to restore a normal rhythm to the fibrillating ventricles using manual massage, artificial ventilation, and intracordial procaine HCl, 15 mg/kg.

*Comment.* Ectopic rhythms may arise following the depression of the normal pacemaker or as a result of impaired intracardiac conduction. The administration of epinephrine may produce this effect reflexly through pressor-vagal reflexes with increase in vagal tone. Changes in vagal tone accompanying the phases of respiration are regarded as the cause of sinus arrhythmia. The increase in cardiac rate following procaine HCl, the absence of sinus arrhythmia, and the failure to demonstrate an increase in vagal tone following epinephrine injection all point to an anti-vagal action of procaine HCl in these experiments.

Burstein *et al.*[3] have reported that by the administration of procaine HCl (5 mg/kg) intravenously, cyclopropane-epinephrine induced ventricular fibrillation may be prevented from occurring, and that the fibrillating heart may be restored to a normal sinus rhythm. We feel that their own observations

[3] Burstein, C. L., Marangoni, B. A., DeGraff, A. C., and Rovenstine, E. A., *Anesthesiology*, 1940, **1**, 167.

do not justify their conclusions, and our results are in disagreement on both points. Not only have we failed to show a protecting effect by procaine HCl, but like Stutzman *et al.*,[4] we have failed to revive the fibrillating heart by cardiac massage and the intracordial injection of 15 mg/kg of procaine HCl.

Allen *et al.*[5] have demonstrated a protecting action of procaine HCl in doses of 16 mg/kg against cyclopropane-epinephrine induced ventricular tachycardia, and feel that such protection is brought about by depression of the ventricular muscle. In their work the epinephrine and procaine HCl were given together, while in our experiments the procaine HCl preceded the epinephrine by 5 minutes. Our inability to duplicate their results may indicate that any such protection of the myocardium is a transient one, particularly when larger doses of epinephrine are employed.

*Conclusions and Summary.* Intravenous procaine HCl increases cardiac rate and restores a regular sinus rhythm to those hearts showing irregularity during cyclopropane anaesthesia. This may account for the favorable clinical impressions on the use of procaine HCl.

A protecting action against cyclopropane-epinephrine induced ventricular tachycardia and fibrillation with doses of procaine HCl reasonable for prophylactic purposes during anaesthesia could not be demonstrated. In our opinion such protection has not yet been demonstrated by others.

Under the conditions of the experiment the predominating effect of procaine HCl given intravenously was an anti-vagal one.

4 Stutzman, J. W., Allen, C. R., and Orth, O. S., *Anesthesiology*, 1945, **6**, 57.

5 Allen, C. R., Stutzman, J. W., Slocum, H. C., and Orth, O. S., *Anesthesiology*, 1941, **2**, 503.

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## Response of the Syrian Hamster to Intradermal Injection of Modified Newcastle Disease Virus. (17363)

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The adaptation of the California strain (No. 11,914) of Newcastle disease virus to the Syrian hamster with 300 intracerebral serial passages and with 9 intranasal serial passages has been reported.[1] Various subcultures of hamster brain material, up to the 200th[2] intracerebral serial passage, were injected intradermally into 4-week-old hamsters (average weight 28 g). No evidence of Newcastle virus infection was noted, although hamsters injected intracerebrally with the same brain suspension showed typical symptoms. No attempts were made to establish Newcastle disease by intradermal injection from the 200th through the 299th intracerebral passages.

The modified hamster virus was first successfully established intradermally by the inoculation of 0.1 cc of a 10% brain suspension of the 300th intracerebral passage in the abdominal region of four 4-week-old hamsters (average weight 28 g). Simultaneously 4 hamsters of the same age and weight were inoculated similarly with 0.1 cc of a 10% brain suspension of the 14th intranasal passage. Both groups of hamsters showed signs of involuntary motor reaction, evidence of salivation, and paralysis in 5 to 10 days.

1 Reagan, R. L., Lillie, M. G., Smith, D. C., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 293.

2 Reagan, R. L., Lillie, M. G., Hauser, J. E., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 293.

TABLE I.
Intradermal Passage of a Strain of Hamster-Adapted Newcastle Virus.

| Material used | Site of inoculation | Passage No. | No. animals inoculated | No. animals showing symptoms | No. days after inoculation symptoms appeared |
|---|---|---|---|---|---|
| 0.1 cc of 10% suspension of 14th intranasal pasage | Intradermally in abdominal area | 1 | 4 | 4 | 5–7–7–10 |
| | | 2 | 6 | 3 | 3–4–4 |
| | | 3 | 6 | 2 | 5–6 |
| | | 4 | 6 | 4 | 5–6–6–6 |
| | | 5 | 6 | 4 | 5–6–6–7 |
| | | 6 | 3 | 3 | 4–4–4 |
| | | 7 | 6 | 3 | 4–5–5 |
| | | 8 | 6 | 6 | 4–4–4–5–5–5 |
| | | 9 | 4 | 4 | 4–5–5–5 |
| | | 10 | 4 | 4 | 3–4–5–5 |
| 0.1 cc of 10% suspension of 300th intracerebral passage | Intradermally in abdominal area | 1 | 4 | 4 | 5–6–6–10 |
| | | 2 | 4 | 4 | 5–5–5–5 |
| | | 3 | 4 | 4 | 5–6–6–6 |
| | | 4 | 3 | 3 | 3–5–6 |
| | | 5 | 4 | 4 | 3–5–5–6 |
| | | 6 | 4 | 4 | 3–4–5–5 |

These symptoms are similar to those occurring in hamsters infected intracerebrally, although salivation has occurred regularly only in later passages. Brains were removed aseptically from the moribund hamsters of each series, ground with alundum, and diluted to a 10% suspension with physiological saline. One-tenth cc of this suspension from each series was injected intradermally into hamsters. By using this technic, the 300th intracerebral brain material was carried up to 6 passages intradermally, and the 14th nasal brain material was carried up to 10 passages intradermally, as shown in Table I. These subinoculations are being continued at the present time.

To determine the distribution of Newcastle virus in the affected hamsters of each series, blood, liver, spleen, kidneys, lungs, spinal cord, and brain were injected into embryonated eggs. Each organ was removed aseptically, ground with alundum and diluted to a 10% suspension with physiological saline. One-tenth cc of citrated blood and of each organ suspension was injected into the allantoic sac of each of six 8-day embryonated White Leghorn eggs. Because of possible bacterial contamination, the lungs, kidneys, liver, and spleen were treated with streptomycin in the ratio of 1 mg per cc of suspension and with penicillin in the ratio of 500 units per cc of suspension before egg inoculation.

Virus was isolated from the brain and spinal cord of hamsters of each series. Virus from the brain and cord of each series was completely neutralized by positive Newcastle chicken serum but it was not affected by normal chicken serum. Virus was not isolated in embryonated chicken eggs from the blood or other organs of either series.

Day-old chicks were injected intramuscularly with 0.2 cc of a 10% suspension of brain material from each series. All chicks showed symptoms of Newcastle disease from 3 to 10 days after injection. Six-week-old chickens were injected intramuscularly with the same amount of a 10% suspension of brain material from each series but showed no evidence of Newcastle disease.

*Discussion.* In the subinoculations up to the 200th intracerebral hamsters passage, hamsters were not infected intradermally. Upon trial of the 300th intracerebral passage and of the 14th intranasal passage, hamsters were infected intradermally, when brain material was used as the inoculum. The 300th intracerebral passage was carried through 6 subinoculations intradermally, and the 14th intranasal passage was carried 10 subinoculations intradermally.

With hamsters as the test animals, virus neutralization tests were conducted, using brain suspensions of the 3rd intradermal passage of the 300th intracerebral series, and

TABLE II.
Titration of Hamster-Adapted Newcastle Virus in Hamsters.

| Virus | Dilution 10-1 | 10-2 | 10-3 | 10-4 | 10-5 | Titer |
|---|---|---|---|---|---|---|
| Hamster brain 5th intradermal passage originally from 14th nasal passage | *4/4 | 4/4 | 4/4 | 0/4 | 0/4 | $10^{-3.5}$ |
| Hamster brain 3rd intradermal passage originally from 300th intracerebral passage | 3/3 | 4/4 | 2/4 | 0/4 | 0/3 | $10^{-3}$ |

* Numerator denotes No. of deaths.
Denominator denotes No. of animals inoculated.

the 5th intradermal passage of the 14th intranasal series. Specific immune chicken serum completely neutralized the intradermal brain virus from both series, whereas normal chicken serum had no effect.

The virus titered $10^{-3}$ in the first series (300th intracerebral) and $10^{-3.5}$ in the second series (14th intranasal), according to the Reed-Muench calculation.[3] Details are given in Table II. Paralysis of the pharynx, as evidenced by excessive salivation with wetting of the mouth and chin, is now a distinct symptom in hamsters.

It has been observed in other modified forms of this virus that day-old chicks are susceptible, whereas older individuals are resistant to infection.

*Summary.* After the 300th intracerebral and the 14th intranasal hamster passage, Newcastle disease virus, California strain No. 11,914 can successfully infect hamsters by intradermal injection. The virus in the brain material from each series was passed intradermally in hamsters. Virus was isolated from the brain and cord of hamsters of both series upon inoculation of embryonated chicken eggs. The results of the neutralization tests confirm the virus to be that of Newcastle disease. Hamsters infected intracerebrally, intranasally, and intradermally show similar symptoms, such as involuntary motor reactions, excessive salivation from pharyngeal paralysis, general paralysis, prostration, and death.

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[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

## Effect of Oxythiamine on the Growth of Chicks.* (17364)

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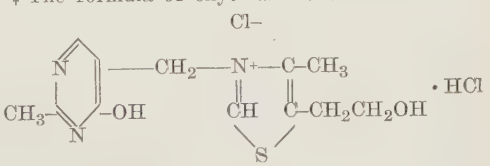
Bergel and Todd[1] reported the synthesis of oxythiamine.‡ They found that 1 mg of this compound possessed no antineuritic ac-

* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by a grant to Cornell University by the Nutrition Foundation, Inc., New York.

† Present address: Department of Biochemistry and Nutrition, Cornell University, Ithaca, N. Y.

[1] Bergel, F., and Todd, A. R., *J. Chem. Soc.*, 1937, 1504.

‡ The formula of oxythiamine is




$C_{12}H_{17}O_2N_3Cl_2S$

tivity when assayed by the bradycardia method using the rat. Soodak and Cerecedo[2] prepared oxythiamine by deamination of thiamine with nitrous acid, and observed that the compound produced a marked toxic effect when fed to mice. The administration of 25 to 50 μg of oxythiamine per day for a 2-week period resulted in the death of young mice maintained on a thiamine-low synthetic diet supplemented with 1 μg of thiamine per day. In this report evidence is presented which shows that oxythiamine is also an active antagonist of thiamine in the nutrition of the chick.

*Experimental.* Four experiments were conducted with day-old White Leghorn cockerels, 10 or 15 per lot. They were housed in electrically heated battery brooders in a room in which the temperature was thermostatically controlled. All chicks were on wire mesh floors to prevent coprophagy. Feed and water were supplied *ad libitum*. The chicks were fed basal diet 653 described by Hill, Norris, and Heuser,[3] modified by adding 100 μg of synthetic folic acid per 100 g of diet and by omitting thiamine. In the first 3 experiments crude casein was used instead of purified casein. In these experiments the basal diet contained between 20 and 25 μg of thiamine per 100 g, as determined by the thiochrome method. The procedure used was that proposed by the Research Corporation Committee on the thiochrome method.[4] The basal diet fed in Experiment 4 in which purified casein was used contained 7 μg of thiamine per 100 g. The chicks were weighed individually at weekly intervals.

*Exp. 1 and 2.* Two preliminary experiments were conducted to study the effects of graded levels of oxythiamine[§] on the growth of chicks to 4 weeks of age. Oxythiamine at the levels of 0.5, 1, 2, and 8 mg was added to 125 μg of thiamine per 100 g of diet. The results indicated that oxythiamine retarded growth at all levels used; the 8 mg level was so highly toxic that only one chick survived to 3 weeks of age. The chicks showed the typical thiamine deficiency symptoms of head retraction, convulsions and inability to stand. Two chicks with these severe symptoms were given 100 μg of thiamine by injection, and within 2 hours they were on their feet and eating. The average 4-week weights of the chicks ranged from 289 g for those receiving 125 μg of thiamine to 182 g for those receiving 125 μg of thiamine plus 2 mg of oxythiamine per 100 g of diet.

*Exp. 3.* This experiment was designed to obtain a quantitative estimate of the effectiveness of oxythiamine as an antagonist of thiamine. The data from Exp. 1 and 2 indicated that the levels of oxythiamine to feed with approximately 125 μg of thiamine per 100 g of diet for the best results were between 1 and 2 mg. Exp. 3 was conducted using an experimental diet containing 120 μg instead of 125 μg of thiamine per 100 g plus 3 levels of oxythiamine. The basal diet contained 5 μg less thiamine per 100 g than the diet fed in Exp. 1 and 2. Eight levels of thiamine were included in order to establish a growth curve. The results of Exp. 3 are presented in Table I.

When oxythiamine was fed in addition to 120 μg of thiamine a marked reduction in growth occurred. By plotting a curve for the effect of varying amounts of thiamine on growth, it was possible to determine the amount of thiamine that was counteracted by the different amounts of oxythiamine. An index was calculated by determining the ratio of the amount of inhibitor to the amount of vitamin antagonized by the inhibitor. The range of the 3 indices obtained in this experiment was 27-39.

*Exp. 4.* This experiment was designed to study the reversibility of this antagonism by additions of thiamine. The basal diet contained purified casein. Increasing levels of thiamine were added to a level of 2 mg of oxythiamine per 100 g of diet. This study was continued for a period of 4 weeks. The

[2] Soodak, M., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1944, **66**, 1988.

[3] Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

[4] Hennessy, D. J., *Cereal Chemists' Bull.*, 1942, **2**, 1.

§ The authors are indebted to Dr. Gustav J. Martin, The National Drug Company, Philadelphia, Pa., for the oxythiamine used in these experiments.

TABLE I.
Inhibitory Action of Oxythiamine on Chick Growth.

| Treatment, μg thiamine | Avg wt 3 wk, g | Avg wt 4 wk, g | Index* |
|---|---|---|---|
| 1. 45† | — (0)‡ | — (0) | |
| 2. 70 | 142 (4) | 161 (4) | |
| 3. 80 | 152 (9) | 218 (7) | |
| 4. 90 | 162 (12) | 251 (10) | |
| 5. 100 | 176 (14) | 264 (13) | |
| 6. 110 | 187 (15) | 250 (15) | |
| 7. 120 | 180 (15) | 264 (15) | |
| 8. 220 | 175 (15) | 266 (14) | |
| 9. 120 + 1 mg oxythiamine | 179 (15) | 233 (14) | 27 |
| 10. 120 + 1.5 mg oxythiamine | 136 (15) | 191 (15) | 34 |
| 11. 120 + 2 mg oxythiamine | 118 (14) | 157 (12) | 39 |

* Index is the ratio of amount of inhibitor to amount of vitamin inhibited.
† Crude casein basal diet contained 20 μg thiamine per 100 g. The levels of thiamine indicate the total thiamine present per 100 g of diet.
‡ Numbers in parentheses represent the surviving chicks of original 15.

TABLE II.
Reversal of Inhibitory Effect of Oxythiamine by Thiamine.

| Treatment, thiamine | Avg wt 3 wk, g | Avg wt 4 wk, g | Index* |
|---|---|---|---|
| 1. 62 μg† | 75 (1)‡ | — (0) | |
| 2. 72 μg | 99 (4) | 86 (1) | |
| 3. 82 μg | 128 (8) | 126 (5) | |
| 4. 92 μg | 140 (7) | 168 (6) | |
| 5. 102 μg | 147 (9) | 194 (9) | |
| 6. 112 μg | 177 (10) | 250 (10) | |
| 7. 122 μg | 185 (10) | 259 (10) | |
| 8. 1 mg | 172 (10) | 246 (10) | |
| 9. 122 μg + 2 mg oxythiamine | 105 (9) | 124 (9) | 49 |
| 10. 147 μg + 2 mg oxythiamine | 116 (10) | 153 (10) | 34 |
| 11. 172 μg + 2 mg oxythiamine | 121 (10) | 172 (9) | 25 |
| 12. 1 mg + 2 mg oxythiamine | 160 (10) | 244 (9) | |

* Index is the ratio of amount of inhibitor to amount of vitamin inhibited.
† Purified casein basal diet contained 7 μg thiamine per 100 g. The levels of thiamine indicate the total thiamine present per 100 g of diet.
‡ Numbers in parentheses represent the surviving chicks of original 10.

results of this experiment are presented in Table II.

The addition of 2 mg of oxythiamine to 122 μg of thiamine caused a 135 g retardation in growth. However, as more thiamine was added to this level of oxythiamine growth was improved. Fifty μg of added thiamine resulted in a 48 g increase, while the addition of 1 mg of thiamine produced normal growth. It is possible that much less than 1 mg of thiamine would have given maximum growth. The inhibitory index for the three levels of thiamine ranged from (25-49).

At the end of the 4th week the chicks of lots 9, 10, and 11 of Exp. 4 were divided into 3 groups. Three chicks from each lot were continued as controls, 3 received injections of 200 μg of thiamine per day into the breast muscle, and 3 received 200 μg of thiamine per day by stomach tube. These chicks were selected to give groups of equivalent weights. All chicks were continued on the diets they had received from the start of Exp. 4. Lot 7 receiving 122 μg of thiamine was continued as the positive control group. The study was carried out for 12 days. The results are summarized in Table III.

The toxicity produced by oxythiamine can be at least partially corrected by additional thiamine. The data show that by injecting or giving thiamine orally a growth response was produced. Injection was somewhat more

TABLE III.
Effect of Parenteral and Orally Administered Thiamine on Oxythiamine-Toxic Chicks.

| | Treatment | Avg initial wt,* g | Avg final wt,† g | Avg change in wt, g |
|---|---|---|---|---|
| 1. | Control | 160‡ | 262 | 102 |
| 2. | Injected§ | 153 | 304 | 151 |
| 3. | Oral‖ | 153 | 290 | 137 |
| 4. | Positive control¶ | 286 | 490 | 204 |

* Weight at 4 wk of age.
† At end of 12-day experimental period.
‡Avg of 8 chicks, rest are avg of 9 chicks.
§ 200 μg thiamine inj. per day into breast muscle.
‖ 200 μg thiamine per day by stomach tube.
¶ Group 7 of Exp. 4 received 122 μg thiamine per 100 g of diet from hatching.

effective than oral administration. In a 12-day period the chicks receiving thiamine gained 35 to 49 g more than the controls, although as should be expected, their growth rate was less than that of the positive controls.

*Discussion.* Oxythiamine produced a thiamine deficiency state in chicks, which could be prevented, or cured to some extent, by administering increased amounts of thiamine. This antagonist was very effective as evidenced by the inhibitory index of 25-49. Although this index is an approximate value it is quite low and comparable to that obtained by Woolley and White[5] for the pyrithiamine: thiamine index in mice of 40. It has been shown by Woolley and White[6] that a low pyrithiamine:thiamine index is obtained with bacteria that require thiamine as the intact molecule. If a microorganism can utilize one or both moieties of the thiamine molecule, the index is 10 to 100 times greater. The chick requires the entire thiamine molecule, thus a low oxythiamine:thiamine ratio is to be expected.

Whether the oxythiamine-thiamine inhibition is a strictly competitive one is doubtful. In Table I it is noted that the chicks in lot 2 averaged 161 g at 4 weeks of age with 4 chicks surviving of the original 15, whereas those in lot 11 grew at the same rate, 157 g, but 12 chicks survived. The same effect is observed in Table II, where chicks receiving low levels of thiamine showed a greater mortality than chicks receiving both oxythiamine and a higher level of thiamine, although the growth rates were similar. (Compare lots 3 and 9, 4 and 11). If simple competition were involved similar mortality would be expected in these lots of chicks.

The requirement of thiamine for White Leghorn cockerels on a purified casein diet is considerably greater than on a crude casein diet. On the purified casein diet the requirement for optimum growth to 4 weeks of age was between 102 and 112 μg of thiamine per 100 g. Whereas, on the crude casein diet the requirement was between 80 and 90 μg per 100 g. The thiamine values obtained by the thiochrome method on replicate samples of each diet were very consistent, although the concentration was low, 0.07 μg per g and 0.2 to 0.25 μg per g, respectively. The explanation for these different requirements is not known, although it may be that the purification process removed from casein some substance that affects the requirement of thiamine. The addition of liver and fish meal supplements to the purified diet produces a significant growth response.[7] It is possible that one of the unknown growth factors present in these materials and crude casein and not in purified casein to any great extent, may affect the requirement of thiamine.

*Summary.* Oxythiamine, the 4'-OH analogue of thiamine, has been shown to be an effective thiamine antagonist in the nutrition of the chick. By increasing the level of oxythiamine fed, the chick weight was decreased until typical thiamine-deficiency symptoms occurred and death ensued. The addition of

[5] Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, **149**, 285.

[6] Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

[7] Unpublished data, Agricultural Experiment Station, Cornell University, Ithaca, N. Y.

large amounts of thiamine to the diet prevented the toxicity due to oxythiamine, and the parenteral and oral administration of thiamine to oxythiamine-toxic chicks tended to overcome the inhibition.

The chick, as well as the mouse, has been shown to be very sensitive to the addition of oxythiamine to the diet. It seems probable, therefore, that oxythiamine is an antagonist of thiamine in the nutrition of all species that require this vitamin as an essential nutrient.

Evidence has been presented which indicates that the thiamine requirement of growing White Leghorn cockerels is greater in the absence of sufficient of the unidentified chick growth factors.

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## The Virostatic and Virucidal Action of α-Haloacylamides on Vaccinia Virus *in Vitro*. (17365)

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During the course of an investigation dealing with the possible antiviral effects of pyrimidine derivatives[1] it was found that amides of 5-aminouracil[1] inhibit the multiplication of vaccinia virus in the tissue culture system previously described.[2] Based on antagonisms demonstrable using *Lactobacillus casei*[3] it was predicted that the climax of this series would be reached with 5-chloroacetamidouracil or amides of 5-aminouracil with other acids having pKa values near that of chloroacetic acid. Actually the activity of chloroacetamidouracil was greater by a factor of 10 or more than the predicted value. Further investigation revealed a high degree of antiviral activity to be a function of all the chloro- and bromoacylamides and dichloroacetamides which were tested including chloroacetamide itself. This paper presents the data on the *in vitro* tests.

*Experimental.* Screening tests with vaccinia virus in chick embryonic tissue culture were carried out as described previously.[2]

*Discussion.* It will be seen (Table I) that bromo- and chloroacylamides usually inhibit growth of the vaccinia virus. The inactivity of the fluoroacetamidouracil (Compound 14, Table I) suggests that an alkylation reaction may be involved in the activity.

When the high degree of activity of 5-chloroacetamidouracil was discovered attention was turned first to the embryonic tissue test system. It was a recognized weakness of the screening test that substances capable of poisoning the embryonic tissue undoubtedly would be expected to prevent the multiplication of the virus.[2] Thus known poisons, such as azide and cyanide, could be shown to inhibit viral multiplication.[2] On the other hand, 5-chloroacetamidouracil was no more toxic to mice than related substances of much lower antiviral potency. It is ineffective against the majority of bacteria, and in reasonable concentration fails to affect the respiration and glycolysis of tissue slices or the glycolysis of washed cell suspensions of *Lactobacillus casei*.†

* These studies were begun at Western Reserve University Medical School (1945) and continued at the Medical College of Virginia (1946-7).

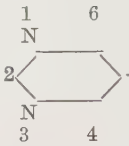
1 Thompson, R. L., Wilkin, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russell, P. B., *Science*, 1949, **110**, 454.

2 Thompson, R. L., *J. Immunol.*, 1947, **55**, 345.

3 Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., and Vander Werff, H., *Annals N. Y. Acad. Sci., in press.*

† We are indebted to Doris Lorz for these determinations.

TABLE I.
Virucidal Activity of Various Haloacylamides.*

| No. | Formula / Compound | Ref. | Conc.† mg per 1 | Increase in virus titer (logarithm) Control | Treated |
|---|---|---|---|---|---|
| 1. | $ClCH_2CONH_2$ | | 100 | 2.03 | —1.16 |
| |  –NHCOR Pyrimidine derivatives | | | | |
| | 2 / 4 / 6 / R | | | | |
| 2. | OH / OH / H / $CH_2Cl$ | § | 10 | 1.04 | —1.30 |
| 3. | OH / OH / $CH_3$ / $CH_2Cl$ | 8 | 100 | 2.24 | —1.30 |
| 4. | $NH_2$ / OH / $CH_3$ / $CH_2Cl$ | 8 | 100 | 2.24 | —1.00 |
| 5. | $CH_3$ / OH / $CH_3$ / $CH_2Cl$ | ‡ | 100 | 2.24 | —1.00 |
| 6. | $NH_2$ / $CH_3$ / $CH_3$ / $CH_2Cl$ | § | 10 | 2.34 | —1.50 |
| 7. | Cl / H / H / $CH_2Cl$ | § | 5 | 2.34 | —1.50 |
| 8. | OH / OH / H / $CHCl_2$ | § | 100 | 2.20 | —0.43 |
| 9. | O / O / H / $CH_2Cl$‖ | § | 10 | 2.75 | —1.79 |
| 10. | $NH_2$ / OH / H / $CH_2Cl$ | § | 10 | 1.40 | —1.50 |
| 11. | $NH_2$ / $NH_2$ / OH / $CH_2Cl$ | § | 100 | 2.09 | —1.00 |
| 12. | OH / OH / H / $CHBrCH_2CH_3$ | § | 100 | 1.89 | —1.84 |
| 13. | OH / OH / H / $CHBrCH_3$ | § | 10 | 2.03 | —1.16 |
| 14. | OH / OH / H / $CH_2F$ | § | 100 | 2.33 | 2.00 |
| 15. | OH / OH / H / $CH_2CH_2Cl$ | § | 100 | 1.85 | 2.02 |
| |  –$NHCOCH_2Cl$ Anilides | | | | |
| 16. | H | 7 | 10 | 2.75 | —1.79 |
| 17. | *p* Cl | 5 | 10 | 1.84 | —1.82 |
| 18. | *p* MeO | 5 | 5 | 1.57 | —1.93 |
| 19. | *p* $NO_2$ | 5 | 5 | 1.57 | —1.93 |
| 20. | *p* $H_2NSO_2$ | 6 | 10 | 2.20 | —1.50 |
| 21. | *p* $H_2NCO$ | 6 | 10 | 2.12 | —2.00 |
| 22. | *p* $CH_3$ | 6 | 100 | 1.76 | —2.16 |
| 23. | 2′Cl 5′Cl | § | 100 | 1.76 | —0.33 |
| 24. | *o* $NO_2$ | 5 | 100 | 1.76 | —1.28 |
| 25. | *m* $NO_2$ | 5 | 5 | 1.57 | —1.93 |

* Determined as described by Thompson.[2]

† The minimal effective concentration is given.

‡ We are indebted to Prof. Alex. R. Todd for this substance.

§ These hitherto undescribed compounds were prepared in The Wellcome Research Laboratories.

‖ 1,3-Dimethyl derivatives.

5 Beckurts, H., and Frerichs, G., *Arch. Pharm.*, 1915, **253**, 233.

6 Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1917, **39**, 2429.

7 Meyer, P. J., *Ber.*, 1875, **8**, 1153.

8 Russell, P. B., Elion, G. B., and Hitchings, G. H., *J. Am. Chem. Soc.*, 1949, **71**, 474.

When the structure of chloromycetin (Chloramphenicol) (IV, Fig. 1) became known,[4] the relationship of this dichloroacetamide to such compounds as 5-chloroacetamidouracil (I, Fig. 1), 5-dichloroacetamidouracil (II) and α-chloro-*p*-nitroacetanilide (III) became apparent. The previous work suggests that the virucidal center of chloromycetin resides in the dichloroacetamide linkage and that all haloacylamides are potentially virucidal. It suggests the possibility that some of the simpler haloacylamides may find practical application in the sterilization of

[4] Rebstock, M. C., Crooks, H. M., Jr., Controulis, J., and Bartz, Q. R., 115th Meeting Am. Chem. Soc., San Francisco, Calif., 1949. Abstracts 9K.

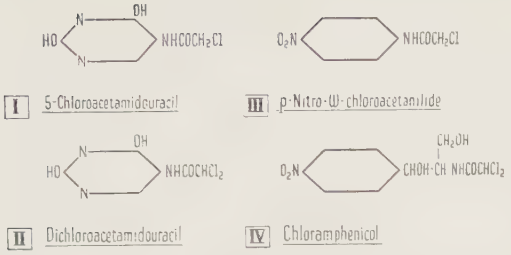



FIG. 1.

substances from the standpoint of viral contamination.

These findings suggest that the tissue culture system when properly controlled is capable of the demonstration of active virucidal chemical groupings. Virucidal substances discovered in this way may be thought of as comparable to bactericidal substances discovered in the usual *in vitro* bacterial growth systems. The relationship between such substances and useful chemotherapeutic agents may be as uncertain in the one biological system as it is in the other.

*Summary.* Several chloro- and bromoacylamides were demonstrated to inhibit multiplication of the vaccinia virus in chick embryonic tissue. 5-Dichloroamidouracil was found to be inhibitory whereas 5-fluoroacetamidouracil was inactive.

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## Seasonal Variation in Human Fasting Blood Sugar Levels.* (17366)

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During the course of a study on human subjects involving the effect of the breakfast meal on carbohydrate metabolism,[1] seasonal variation in fasting blood sugar levels was noted.

Work done in this laboratory over a 2 year period on 9 women gave a total of 170 fasting blood sugar determinations. When these values were averaged by months, the results shown in Fig. 1 were obtained. The fasting values during the 2 year period were within the range given by Peters and Van Slyke.[2] However, they showed marked seasonal differences. Blood sugar concentration rose progressively with the colder months of the year and were definitely higher in the winter than during the summer months.

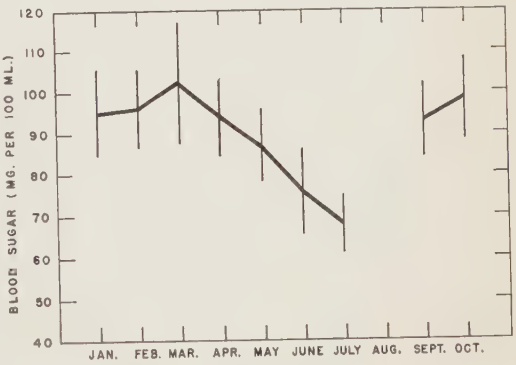


FIG. 1.

Average blood sugar values by month. Standard deviations indicated by vertical lines.

The subjects used in this study were 9 women laboratory workers ranging in age from 28 to 48 years They were of average weight and height and moderately active. They were considered to be in good health although no physical or medical examinations were made.

Periodic blood counts and urine analyses were normal. At no time did any of the subjects participate in an experiment when suf-

* This research was done as part of a project supported by an allotment made by the Secretary of Agriculture from Special Research Funds (Bankhead-Jones Act of June 29, 1935).

1 Orent-Keiles, Elsa, and Hallman, Lois F., U.S.D.A. Circular, 1949, No. 827.

2 Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Vol. 2, Chap. III, Williams and Wilkins, 1946.

fering from a cold or similar indisposition. Neither were tests made during menstruation. By coincidence none of the subjects were smokers.

Samples of blood were taken after 15 hours' fasting and collected from a fingerprick into a paraffin well. Folin's micro method[3] was used for the glucose determinations, using 1.6% sodium carbonate in place of the sodium cyanide-sodium carbonate solution as recommended by Klendshoj and Hubbard.[4] Readings were made on a Beckman spectrophotometer at 5200 Å. All measurements and readings throughout the study were done by the same analyst. Periodic standard sugar curves were made to check the solutions and the instrument. A search of the literature revealed only 2 observations[5,6] which suggest a relationship of season to concentration of blood sugar in human subjects.

The results obtained in the study reported here present the question whether these seasonal differences are due to variation in the quality or quantity of the food ingested or difference in the individual metabolism, or both, at different seasons of the year.

[3] Folin, O., and Svedberg, D., *J. Biol. Chem.*, 1930, **88**, 85.

[4] Klendshoj, Niels C., and Hubbard, Roger S., *J. Lab. Clin. Med.*, 1939-40, **25**, 1102.

[5] Johnson, Buford, J., *J. Comp. Psychol.*, 1922, **2**, 155.

[6] Strouse, S., *Arch. Int. Med.*, 1920, **26**, 758.

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## Hearing in Guinea Pigs Deficient in the Anti-Stiffness Factor.* (17367)

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This research was outlined to establish whether a diet deficient in the anti-stiffness factor[1-5] produced diminution in the hearing ability of guinea pigs. Responses of guinea pigs to sound are various. The most frequently observed reflex is the well-known ear-flick which, when well developed, is a folding back of the ear against the side of the head, but in diminished form is a mere quiver of the edge of the ear. This type of response was chosen as the most clear-cut obtainable to establish the hearing range of guinea pigs, although certain animals gave evidence by jerking or movement of whiskers that they heard a little beyond the limits set by the ear-flick.

Stimuli were furnished by a General Electric audio-signal generator combined with a loud speaker and key (borrowed through the kindness of Prof. E. A. Yunker, Department of Physics, Oregon State College). Free response was obtained by placing the unconfined animal upon the table, closely facing the loud speaker. Each guinea pig was exposed to a series of signals from a frequency of zero to a frequency of 17,000 cycles per second. The range in pitch to which ear-flick response was given was found to be 150-16500 cycles per second. Fluctuations in electric current from time to time produced slight variations in response.

The 68 experimental animals used in this research had been fed a diet deficient in the anti-stiffness factor for at least 11 months before the testing started. This diet consisted of 18-20% skim milk powder in water, to which was added copper and iron salts and the following assortment of vitamins: The

* Supported in part by a grant from General Research, Oregon State College.

[1] Wulzen, R., and Bahrs, A. M., *Physiol. Zoology*, 1936, **9**, 508.

[2] Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, 1941, **133**, 500.

[3] van Wagtendonk, W. J., *J. Biol. Chem.*, 1944, **155**, 337.

[4] Simonsen, D. H., and van Wagtendonk, W. J., 1947, **170**, 239.

[5] Folkers, K., *Chem. and Physiol. of Growth*, 1949, 82-86.

salts and ascorbic acid, 10 mg per animal, were added to the morning feeding. The fat soluble and the water soluble vitamins were added alternately to the evening feeding, allowing 2 cc of the solutions for each animal.

| | Vitamin solutions | |
|---|---|---|
| Water soluble | Thiamine hydrochloride | 0.2 g |
| 1 liter | Riboflavin | 0.5 |
| | Pyridoxine hydrochloride | 0.1 |
| | Nicotinic acid | 1.0 |
| | Calcium pantothenate | 0.1 |
| | Inositol | 10.0 |
| | p-Amino benzoic acid | 2.0 |
| | Choline hydrochloride | 50.0 |
| | Folic acid | 0.003 |
| | Biotin concentrate | 3 drops |
| Fat soluble | Beta carotene | 20 cc |
| 1 liter | Viosterol | 10 cc |
| | Alpha tocopherol | 0.1 g |
| | 2-$CH_3$-1, 4-naphthaquinone | 0.1 g |

The 68 deficient animals were compared with 68 non-deficient stock guinea pigs which had been fed a diet of rolled barley, with abundant green feed daily. Both groups of animals were bedded in wheat straw and provided with iodized salt and water.

Both stock and deficient animals were subjected to frequent hearing tests over a period of 7 months. In both groups it was found that individuals differed from one another in hearing to some degree, but this difference involved almost exclusively the lowest extent of the hearing range, up to about 2000 cycles per second. The upper limit of hearing with apparently normal animals was very regularly somewhat above 10000 cycles per second.

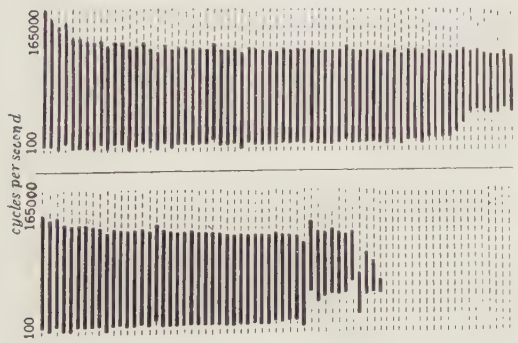


FIG. 1.

*Upper* series represents hearing range of 68 guinea pigs fed stock diet. *Lower* series represents hearing range of 68 guinea pigs fed deficient diet. Length of individual lines indicates range in pitch of audio-signals from 100 to 16,500 cycles per second. Solid portion of line indicates actual range of each animal. Note (1) that no stock animal had seriously diminished hearing range; (2) 19 deficient animals gave no auditory response.

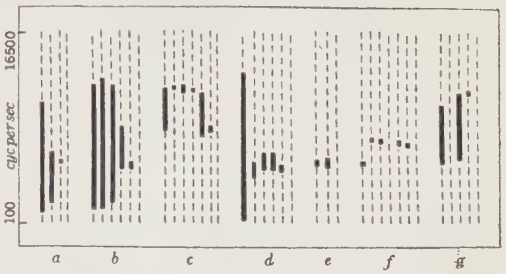


FIG. 2.

Seven individual cases of deficient guinea pigs showing recession of hearing during an experimental period. Length of individual lines indicates range in pitch of audio-signals, 100 to 16,500 cycles per second. Solid portion of line indicates actual hearing range of deficient animal on day tested. Each group of lines represents successive hearing responses of one animal. The decline of each animal to final deafness is shown by an overall decrease in length of solid lines. Final dotted line in each group shows entire absence of hearing response, which was proved by many subsequent tests to be permanent deafness.

Intervals in days between testing dates were as follows: (a) 30, 30, 23, (b) 30, 45, 23, 14, 8, (c) 30, 45, 10, 4, 3, 16, (d) 30, 45, 23, 10, 4, (e) 30, 45, (f) 30, 35, 10, 4, 3, 17, (g) 30, 35, 17, 16.

The 68 non-deficient animals showed normal range of ear-flick response at each test over the 7-month period of observation, the most restricted range being 2,000-10,000 (Fig. 1, upper). Of the 68 deficients tested, 11 never gave ear-flick response at any time during the 7-month period. It should be noted that these animals had been on deficient diet for at least 11 months before the first test. By the end of the 7-month experimental period, 8 more guinea pigs had become deaf (gave no ear-flick response), making a total of 19 deaf animals (Fig. 1, lower). The advancing deafness of 7 of these individuals is illustrated in Fig. 2.

Statistical analysis was made of data on hearing ability of 56 stock animals and 64 deficient animals. The average ranges (highest frequency at which ear-flick was obtained less lowest frequency at which ear-flick was obtained) are given in Table I. The analysis of variance shows that the animals which were fed with the same diet differed from one another in range of response to audio-signals ($F = 34.75$ with 118 and 429 degrees of

TABLE I.
Average Range of Hearing.

| | Stock | Deficient |
|---|---|---|
| No. of animals | 56 | 64 |
| No. of measurements | 176 | 373 |
| Avg range | 10,630 | 7,328 |

freedom). It also shows that animals fed stock diet had significantly wider range of response to audio-signals than those fed deficient diet ($F = 18.34$ with 1 and 118 degrees of freedom).

*Summary.* Guinea pigs fed a diet deficient in the anti-stiffness factor may ultimately develop inability to respond with the ear-flick to auditory stimuli. At the conclusion of the experiment, 28% of the deficient animals were deaf according to the ear-flick test, in contrast to 0% among the stock animals. It was possible to trace developing deafness according to the ear-flick test in a considerable number of animals maintained on a deficient diet during the experimental period of 7 months.

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## Evidence that Copper Acetate Induces Ovulation in the Rabbit by Direct Stimulation of the Adenohypophysis. (17368)

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Copper salts induce ovulation in the estrous rabbit,[1,2] a species which normally ovulates only after copulation. The natural mating stimulus, which results in the release of luteinizing hormone from the adenohypophysis, involves the hypothalamus and the pituitary stalk[3] and includes both adrenergic and cholinergic components.[4]

Bischoff[5] suggested that copper activated the hypophysis by toxic stimulatory effects on the nervous system. The idea appeared to be confirmed by the results of Brooks,[3] who found that copper failed to induce ovulation in rabbits whose hypophyseal stalks had been severed, and Harris,[6] who reported that very weak dosages of copper acetate injected directly into the third ventricle stimulated the ovulatory response.

We were led to doubt that copper-induced ovulation is purely a nerve-stimulation phenomenon when dibenamine and atropine, in dosages adequate to block the copulation stimulus from reaching the hypophysis,[4,7] failed to prevent copper acetate from activating the release of an ovulatory surge of LH.[8] It therefore seemed desirable to test the hypothesis that copper might exert its stimulatory action directly upon the anterior pituitary.

Sexually mature female rabbits ranging in weight from 2.5 to 4.3 kg were employed in this study. To insure an estrous condition, each animal was treated with 85 $\mu$g estradiol benzoate on 2 successive days prior to copper administration, for ovulation is not induced by copper in anestrous rabbits.[9] A control series of 10 females revealed that the estrogen alone would not stimulate LH release. The hypophysis was approached parapharyngeally as in earlier studies,[10,11] and 4 attempts were made at 5 or 10 minute intervals to inject a total of 0.15 ml of 0.1% copper acetate, buf-

1 Fevold, H. L., Hisaw, F. L., and Greep, R., *Am. J. Physiol.*, 1936, **117**, 68.

2 Emmens, C. W., *J. Endocrin.*, 1940, **2**, 63.

3 Brooks, C. Mc., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **20**, 525.

4 Sawyer, C. H., Markee, J. E., and Townsend, B. F., *Endocrinology*, 1949, **44**, 18.

5 Bischoff, F., *Am. J. Physiol.*, 1938, **121**, 765.

6 Harris, G. W., *J. Physiol.*, 1941, **100**, 231.

7 Sawyer, C. H., Markee, J. E., and Everett, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 670.

8 Sawyer, C. H., and Markee, J. E., unpublished.

9 Dury, A., and Bradbury, J. T., *Am. J. Physiol.*, 1943, **139**, 135.

10 Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Endocrinology*, 1946, **38**, 345.

fered with 0.1% sodium acetate,[12] directly into the pars distalis.

In 12 of the 20 experimental animals which survived this operation for 48 hours the copper did not reach the hypophysis; the hypophyseal capsule remained intact. None of these 12 revealed ruptured or hemorrhagic follicles at autopsy. This group included 5 injected with copper acetate in an India ink suspension, and the ink was found in the retropharyngeal region below the sella and in the cavernous sinuses. Of the 8 whose hypophyses actually received copper, 6 ovulated. Two of the 6 had hypophyses penetrated to the top in such a way that some of the copper could possibly have reached the hypothalamus, but in the other 4 no such condition existed. Three of the 8 injected hypophyses were entered with the copper-ink medium and of these, 2 ovulated; but in all 3 most of the ink was located inferior to the sellar region, none above the diaphragma sellae. The effects of mechanical trauma were controlled by a series of 7 animals into each of whose hypophyses a bipolar electrode was inserted,[10] and a second series of 23 rabbits whose hypophyses were injected with acetylcholine-eserine solution.[11] None of the former and only one of the latter ovulated.

At first glance our results, ovulation in 75% of 8 rabbits, on intrapituitary injection of copper, might seem unconvincing compared with Harris' 80% of 13 animals on injection of copper into the third ventricle, especially since Harris used a dosage only a third as great as ours. However, most of our injected material backed out of the sella, away from the hypophysis and hypothalamus, while Harris' copper could have reached and stimulated the hypophysis directly rather than the hypothalamus as he assumed. His copper, deposited in the third ventricle, may well have been picked up by the proximal capillary plexus of the hypophyseal portal system and carried by the portal veins directly to the adenohypophysis. Brooks' failure to induce ovulation with copper in stalk-sectioned animals is difficult to explain in terms of a direct-action hypothesis, since presumably an adequate arterial blood supply to the adenohypophysis remained intact after his operation. The possibility does exist, however, since he could not test them by the mating response and since he supplied no estrogen, that his copper-treated stalk-sectioned animals were anestrous.

To summarize, the intrahypophyseal injection of 1/100th of the systemic dose of copper acetate, an agent whose effect on stimulating the release of hypophyseal LH is not blocked by anti-adrenergic or anti-cholinergic drugs, has led to ovulation in 6 out of 8 rabbits. It is, therefore, concluded that at least part of the copper effect is a direct stimulation of adenohypophyseal cells.

[11] Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Endocrinology*, 1946, **38**, 345. 1948, **2**, 117.

[12] Dury, A., and Bradbury, J. T., *Am. J. Physiol.*, 1942, **135**, 587.

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## Reduction of Potassium Tellurite by Living Tissues.* (17369)

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The property of soluble colorless tetrazolium salts to form insoluble red to purplish formazans as reduction products can be used for the demonstration and microscopic localization of reducing activity in living tissues.[1-4]

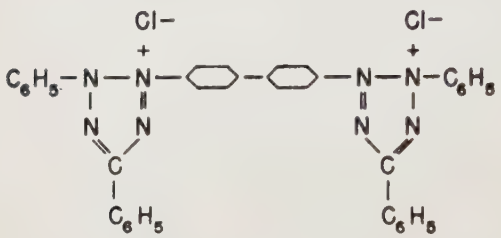
* This work was done under a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

[1] Mattson, A. M., Jensen, C. O., and Dutcher, R. A., *Science*, 1947, **106**, 294.

[2] Pratt, R., and Dufrenoy, J., *Stain Technology*, 1948, **23**, 137.

Potassium tellurite introduced into bacteriology by Klett[5] and later utilized by Conradi and Troch[6] for the isolation of diphtheria bacilli, was found to be a suitable reagent for this purpose since reduced insoluble tellurium imparts a distinct black color. A similar reagent had been previously employed by Hasegawa[7] for the determination of the germinability of cereal seeds.

*Method.* A 0.1% solution of potassium tellurite C.P. in M/10 phosphate buffer pH-7.4 gave reproducible and consistent results. For comparison, a saturated solution of "neotetrazolium chloride" obtained from Pannone Chemical Company, Farmington, Conn. in physiological saline solution or phosphate buffer was likewise used. The formula of this compound is:



*Results and discussion.* 24 hour cultures of various bacteria (*E. coli, S. typhimurium, S. oranienburg, S. montivideo, S. typhosa, Shigella alkalescens, Proteus morganii, Proteus vulgaris, Pseudomonas aerugenosa, B. subtilis, Staphylococcus, Streptococcus viridans, C. diphtheriae*) grown in 2% peptone water or nutrient broth reduced both "neotetrazolium chloride" and potassium tellurite. 0.2 cc of the reagents were added to 3 cc of the culture medium. A strong reduction was also seen by several strictly anaerobic bacteria, (*C. tetani, C. botulinum, C. perfringens*) grown in tryptose broth, regardless of whether the cultures after the reagent had been added were returned to strictly anaerobic condition or left outside in the air. In general, the reduction of potassium tellurite required several hours while that of "neotetrazolium" was apparent within 30-60 minutes. Under the microscope, many of the bacteria as well as the cocci showed similar inclusions with both reagents. The ones following treatment with "neotetrazolium"[4] were larger and coarser, but their general shape and distribution was alike with both preparations.

Suspensions of fresh[8] and of air-dried yeast[9] reduced tetrazolium salts as well as potassium tellurite. Under the microscope, many yeast cells showed fine and coarse granules of similar morphology with both preparations.

Material from various kinds of exudates from human patients were suspended in potassium tellurite and "neotetrazolium chloride" solutions at 37°C. In a considerable amount of polymorphonuclear leucocytes, reducing activity was revealed by the occurrence of purplish red and black granules respectively, of similar appearance and distribution. Thin tissue pieces from rat and rabbit organs as well as from operatively removed human material were suspended in potassium tellurite and "neotetrazolium chloride" solutions and kept at 37°C. Within 15 to 30 minutes, a discoloration took place increasing in intensity within the next hours. The extent of discoloration was similar with both reagents. Kidney tissue, *e.g.*, showed the strongest reaction while intestines reacted only moderately and fat tissue very weakly. A more consistent and deeper penetration of the tissues was however noted with potassium tellurite.

The distribution of reduced tellurium can be studied in frozen sections prepared from formalin fixed tissues cut at 15 to 25 $\mu$ that had been immersed for 8 to 12 hours in the potassium tellurite solution previous to fixation. Paraffin embedding may also be employed but has so far given inferior results. Particularly satisfactory preparations were obtained with kidney tissue of animal as well

[3] Straus, F. H., Cheronis, N. D., and Straus, E., *Science*, 1948, **108**, 113.

[4] Antopol, W., Glaubach, S., and Goldman, L., *Public Health Reports*, 1948, **63**, 1231.

[5] Klett, A., *Zeitschr. Hyg.*, 1900, **33**, 137.

[6] Conradi, H., and Troch, P., *Munch. Med. Wochensehr*, 1912, **59**, 1652.

[7] Hasegawa, K., *Japanese J. of Botany*, 1936, **8**, 1.

[8] Kuhn, R., and Jerchel, D., *Ber der Deutchen Chem. Ges.*, 1941, **74B**, 949.

[9] Gunz, F. W., *Nature*, 1949, **163**, 98.

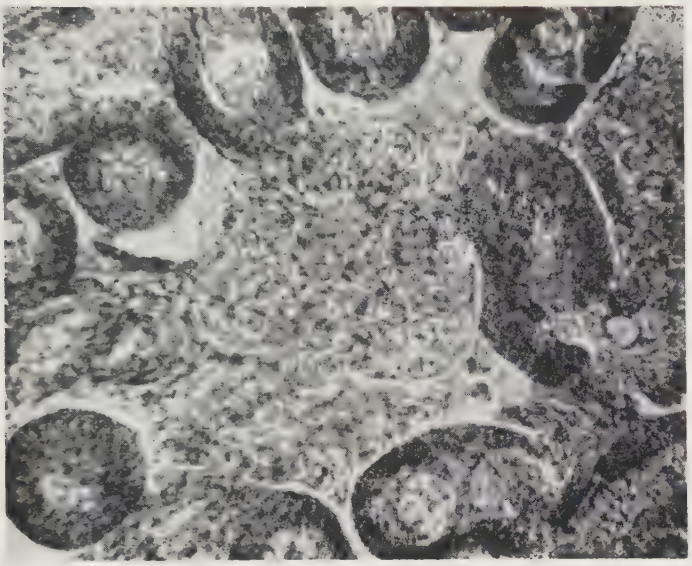


FIG. 1.
A frozen section from a rabbit kidney showing tellurium deposition in the proximal convoluted tubules. × 450.

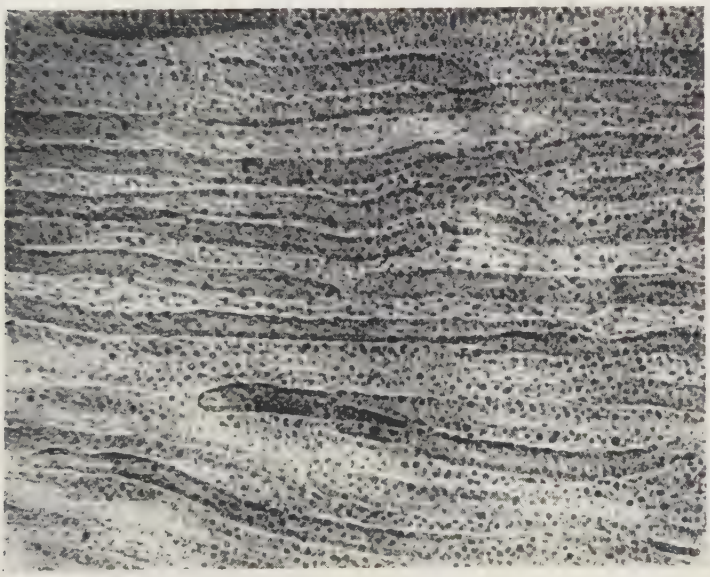


FIG. 2.
A frozen section from rabbit kidney showing tellurium deposition mainly in ascending limbs of Henle's loops in the outer zone of the medulla. × 150.

as human origin. In the cortex, the proximal and distal convoluted tubules, as well as ascending limbs of Henle's loops, showed deposition of tellurium (Fig. 1). Within the outer zone of the medulla, ascending and descending limbs of Henle stained selectively (Fig. 2) while in the innermost portion of the medulla, occasional collecting tubules revealed reducing activity. The intensity of the staining reaction varied considerably from field to

field. In general, however, proximal convoluted tubules and ascending limbs of Henle's loop reacted strongest.

Intravital reduction of tellurium in the kidney could also be demonstrated with potassium tellurite, although the reaction was less extensive. Two groups of 6 adult rats received a daily intraperitoneal injection of 0.8 cc of "neotetrazolium chloride" and potassium tellurite solution, respectively, for 16 days. On gross examination, the cortical portion of the kidney was stained. Microscopically, reduction took place only in the proximal convoluted tubules. The depositions of tellurium as well as of the formazan were considerably less intense than in supravitally stained kidney tissue. With potassium tellurite there was however very little reduction in the liver, while considerable dye deposition took place in the parenchymal cells with "neotetrazolium chloride".[4] The deposition of tellurium in the kidney took place in such a manner as to suggest a localization within large droplets and rods closely simulating the microscopic appearance of kidney sections that had been specifically stained for mitochondria. On chemical examination, Schneider found various enzymes including succinic dehydrogenase to be associated with the mitochondria or large granule fraction from rat liver and kidney homogenates.[10]

The conditions under which potassium tellurite is reduced are closely similar to those under which "neotetrazolium chloride" is acted upon. Boiling, alcohol, acetone, and formalin fixation destroy the reducing ability of animal tissue for both reagents. Antopol and coworkers[4] have noted that in the test tube, cysteine and glutathione at pH-7.0 reduce "neotetrazolium chloride" in contrast to cysteine and methionine, thus indicating the importance of active SH groups. An identical behavior was found with potassium tellurite. Correspondingly, treatment with sulfhydryl reagents prevented the reduction of tetrazolium salts by fresh tissue. Similarly, pieces of fresh kidney immersed for one hour in a 0.1% solution of iodocetamide[11] and then incubated up to 20 hours in potassium tellurite or "neotetrazolium" remained completely unstained. Control tissue immersed first in physiological saline solution and then treated with the reagents showed a very strong reaction. There is however considerable lack of specificity for inhibitors of the reduction of tetrazolium salts by living tissues.[12] This would indicate that a number of reducing enzymes, acting possibly on various materials within the cell could be involved in the reducing process.

*Summary.* Reducing activity in living tissue can be demonstrated with the aid of potassium tellurite. Under the microscope, the sites of reduction are indicated by insoluble dark tellurium. Various bacteria, including strictly anaerobic ones as well as yeast and leucocytes from human material, reduced posassium tellurite. Among different mammalian tissue examined, kidney gave the most constant result, permitting histochemical localization in certain portions of the nephron. Active SH groups apparently are essential for the reduction of potassium tellurite by living tissues.

The writer appreciates the technical assistance of Miss Esther Levenkron.

[10] Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

[11] Barron, E. S. G., and Singer, T. S., *J. Biol. Chem.*, 1945, **157**, 221.

[12] Fred, R. B., and Knight, S. G., *Science*, 1949, **109**, 169.

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# Amino Acid Balance at Super-normal Dietary Levels.* (17370)

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*From The Grange Company, Modesto, Calif.*

It has been pointed out that the same relative proportions of indispensable amino acids to each other must be established to secure the maximal gain possible by the chick on a particular diet, whether the amino acids are supplied as mixtures made up from the single amino acids, as hydrolyzed proteins, or as intact proteins. These findings were based primarily upon protein levels at an approximate optimal value of 20% of the diet.[1] Recently it was shown by Grau that the lysine required to supplement sesame protein for maximal growth of the chick at any protein level from 5 to 30% of the diet was almost directly proportional to the protein level.[2] The present studies were conducted to investigate the relation of dietary protein level to methionine requirement of the chick.

*Experimental.* S. C. White Leghorn, New Hampshire and New Hampshire-Barred Rock Cross cockerel chicks, were fed for one or 2 weeks a practical starting mash, then closely selected for weight and vigor, divided into groups of 10 to 15 each, and placed on the experimental diets. The soybean meals used were solvent-process meals of known good quality, and with only very slight urease activities. The diets were made up as indicated in Table I.

TABLE I.
Composition of Basal Diets.*

| | I | II |
|---|---|---|
| Soybean meal | 408 g | 617 g |
| Bonemeal | 50 g | 50 g |
| Limestone | 10 g | 10 g |
| Paper pulp | 20 g | 0 g |
| Salt | 10 g | 10 g |
| Animal protein factor conc. | 11 g | 11 g |
| Cystine | 1.1 g | 1.1 g |
| Riboflavin | 4.4 mg | 4.4 mg |
| Ca. pantothenate | 11.0 mg | 11.0 mg |
| Niacin | 44.0 mg | 44.0 mg |
| Thiamin | 4.4 mg | 4.4 mg |
| Choline chloride | 660 mg | 660 mg |
| Vit. E | 11.0 mg | 11.0 mg |
| Vit. A, U.S.P. units | 11,000 | 11,000 |
| Vit. D, A.O.A.C. units | 1,100 | 1,100 |
| Glucose to | 1 kg | 1 kg |

* Vitamin quantities indicated are in addition to those present in the soybean meal.

† Supplied by Lederle Laboratories through the kindness of Dr. T. H. Jukes.

The chicks were kept for 2 weeks on the experimental diets. Supplements of DL-methionine, and results obtained are indicated in Table II.

Recent analyses of soybean meals for methionine, and, in particular, one thorough study,[3] have shown that modern soybean meals cannot be expected to provide much more than approximately 1.5% methionine, as calculated on the crude protein (% N × 6.25) basis. More than 0.10% but not more than 0.20% added methionine was needed to permit maximal gain at the 20% protein level. The addition of 0.20% methionine brought the total up to 0.50%, which is the previously estimated minimum requirement standard for chicks.[1] This requirement had been estimated by the use of S. C. White Leghorn chicks. The present data show that this estimate holds equally well for New Hampshire and New Hampshire-Barred Rock Cross chicks. On the other hand, McGinnis and Evans[4] have suggested that New Hampshire chicks may require less methionine than White Leghorns.

The 30% protein diets were not completely supplemented by an addition of 0.20% methionine. It is probable that very nearly 0.30% added methionine, (0.75% total methionine) but not more, was required to permit maximal rates of gain with the 30% protein diets. Evidently, the amount of total methionine needed to support the maximal rate of gain was proportional to the total protein level.

* Presented at the Informal Poultry Nutrition Conference, Detroit, April, 1949.

1 Almquist, H. J., *J. Nutrition*, 1947, **34**, 543.

2 Grau, C. R., *J. Nutrition*, 1948, **36**, 99.

3 Kuiken, K. A., and Lyman, C. M., *J. Biol. Chem.*, 1949, **177**, 29.

4 McGinnis, J., and Evans, R. J., *J. Nutrition*, 1947, **34**, 725.

TABLE II.
Relation of Protein and Methionine Levels to the Rate of Gain in Chicks.

| Protein in diet, % | Methionine added to diet, % | Total methionine in diet, % | Daily rate of gain | | |
|---|---|---|---|---|---|
| | | | S.C. White Leghorn, % | New Hampshire, % | New Hampshire-Barred Rock cross, % |
| 20 | 0 | .30 | 6.3* | 7.1* | 7.2 |
| 20 | .10 | .40 | 6.7 | 7.6* | 7.7 |
| 20 | .20 | .50 | 7.0* | 8.0* | 7.9 |
| 20 | .30 | .60 | 7.0 | 7.9* | 7.9 |
| 30 | 0 | .45 | 6.2* | 8.2* | — |
| 30 | .10 | .55 | — | 8.4* | — |
| 30 | .20 | .65 | 6.7 | 8.5* | 8.2 |
| 30 | .30 | .75 | 7.2* | 8.6* | 8.5 |
| 30 | .40 | .85 | 7.2 | 8.6* | 8.4 |

* Average growth rate of duplicate pens.

These results are analogous to those with sesame meal and lysine.[2]

The results discussed above seem sufficient to justify an extension of the concept previously stated by the writer,[1] that the proportions of indispensable amino acids to each other remain relatively the same for maximal efficiency of utilization under varying conditions of level and source of the amino acids. This concept now includes levels of protein intake considerably above what is usually considered the normal requirement for maximal rate of gain.

Since maximal protein efficiency, as indicated by growth rate, is closely related to proportions of amino acids in the diet, even at super-normal protein levels, it would appear, as previously suggested,[1] that "the proportions of amino acids reaching the synthetic regions in the animal are determined largely by the proportions in the diet." The balance of indispensable amino acids in the nitrogenous increments reaching these cells is more important than the gross protein intake.

Amino acid imbalance in chick diets is not fully correctable by feeding more of the same imbalanced protein. This fact has a bearing on chick assay diets for "animal protein factor," in which approximately 70% soybean meal is used. Even with best qualities of soybean meals, these diets are methionine deficient, and when a material is added which contains both "animal protein factor" and an effective quantity of methionine, as in the case of fish meals, the growth response observed is a resultant of a dual supplementation.

*Summary.* The methionine requirement of the chick for maximal rate of gain on a 30% protein diet is approximately 0.75% of the diet. This value is in direct proportion of the protein levels to the established methionine requirement of 0.50% in a 20% protein diet.

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## Rapid Measurement of the Oxygen Saturation of Whole Blood Samples with the Millikan Oximeter.* (17371)

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(With the technical assistance of Katherine S. Gullixson.)

*From the Departments of Physiology and Biochemistry, University of Oregon Medical School, Portland, Oregon.*

During cardiac catheterization it is frequently desirable to obtain a rapid estimation of blood oxygen saturation in order to orient abnormal locations of the catheter and to determine the uniformity of multiple samples. With this purpose in mind, Groom, Wood, Burchell and Parker[1] have developed a whole blood cuvette oximeter which requires a spec-

cial photocell unit. We have adapted the principles outlined by these workers to the use of the compensated circuit Millikan oximeter. The earpiece of this apparatus is clamped over a plastic tube cuvette. Standardization of the red and infra-red-red bias circuits is obtained by galvanometer settings with the neutral filter and with the cuvette filled with oxygen saturated blood respectively.

*Apparatus*: To utilize the photosensitive area of the Millikan earpiece to the maximum, a length of translucent polythene plastic tubing is looped to pass twice through the earpiece perpendicular to the long axis of the photocell limb. The tubing is compressed slightly by thin bakelite plates. Each plate contains an ovoid window in which a microscope cover-glass has been cemented recessed flush with the inside surface of the plate. The diameter of the window perpendicular to the axis of the tubing is determined by the lateral edges of the lumens of the two segments of the polythene loop lying in close proximity. Such dimensions shield light which would pass laterally to the fluid columns. The ends of the ovoid window are arcs of a circle equal

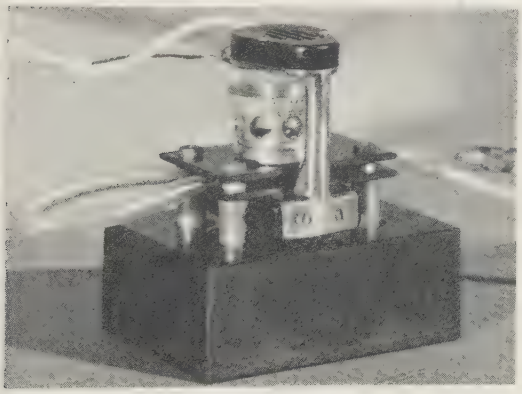

FIG. 1A.
Assembled Cuvette-Earpiece unit. The setscrews controlling cuvette thickness fit into the bakelite base and are not seen.

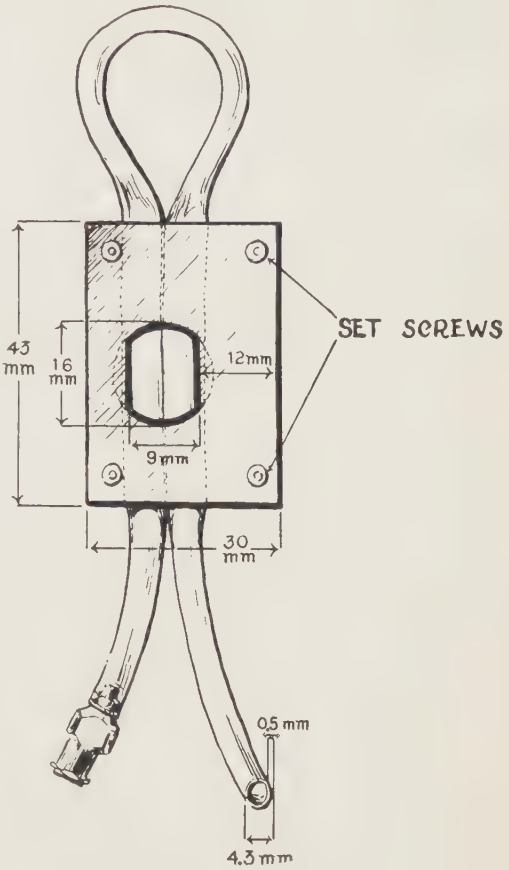


FIG. 1B.
Detail of cuvette light port.

in diameter to the photocell port in the Millikan earpiece. The loop of tubing is compressed to produce a light path distance 1.2 mm through the lumen by a set screw with lock nut at each corner of the bakelite plates. The ovoid window is placed asymmetrically in the plates so that the earpiece is centered over the window when its upright is against one edge of the cuvette plates and the photocell limb is against two of the set screws. This insures a relatively constant location of the earpiece prior to standardization. The plastic tubing is 4.3 mm in external diameter with walls 0.5 mm thick. The tubing, obtained from an ordinary disposable intravenous infusion set (Baxter), may vary slightly in diameter and translucency without affecting the accuracy of the apparatus. When this tubing is compressed to internal diameters

* Aided by grants from the American Heart Association and the Oregon Heart Association. The oximeter was made available by a generous gift from Mr. Henry Chaney to the Doernbecher Memorial Hospital for Children.

1 Groom, D., Wood, E. H., Burchell, H. B., and Parker, R. L., *Proc. Mayo Clinic Staff Meetings*, 1948, **23**, 601.

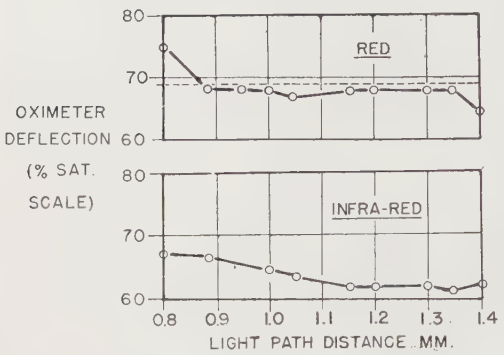


FIG. 2.

Light transmission properties with varying light path distance. At each light path distance the red infra-red bias circuit was standardized with fully saturated blood in the cuvette. Aliquots of a nitrogen desaturated sample of the same blood then produced galvanometer deflection. The lower portion of the diagram shows the galvanometer deflection produced by activation of the infra-red photocell alone. The upper portion of the diagram (RED) shows galvanometer deflection produced by the bias circuit measuring oxygen saturation. The dotted line indicates oxygen saturation of the nitrogenated blood sample as measured by Van Slyke manometer. Between light path distances of 0.88 mm and 1.35 mm a constant half scale galvanometer deflection is produced with desaturated blood having an oxygen capacity of 23.1 volumes %.

shown in Fig. 2, an area of absorbing solution on the order of 120 sq mm is exposed to light. One end of the tubing is fitted with the hub of an 18 gauge hypodermic needle, which has been drilled to larger bore. The set-screws of the cuvette fit into a heavy bakelite base.

A commercial oximeter serves as the recording device and as power unit for the earpiece lamp.† The earpiece utilizes a light filter in the red range and infra-red range rather than the red and green light range of the original Millikan earpiece. The bias circuit of this apparatus results in direct recording of oxygen saturation; such a circuit is described in Millikan's original communication[2] as a single scale oximeter with automatic adjustment for ear thickness. Currents are recorded with a mirror galvanometer (Rubicon 3415). The scale, 10 cm in length, is calibrated directly in percent oxygen saturation from 50% to 100% in 5% scale divisions.

*Procedure.* A warm-up period of 5 to 10 minutes is required for the earpiece circuits. A standard galvanometer deflection of the infrared cell circuit ("green" circuit) alone is set using variable resistance $R_1$ with a standard filter in the light path of the earpiece (Coleman A Filter). The earpiece is clamped firmly in place over the cuvette. A blood sample is drawn by catheter from the patient and saturated with tank oxygen at room temperature. With this saturated blood in the cuvette, the red-infra-red bias circuit is standardized at 100% saturation with variable resistance $R_2$. The oximeter remains stable over a period of hours, but bias circuit standardization may be repeated at intervals. Once standardization is completed, optical arrangements should not be altered by change of position of setscrews, earpiece screw or earpiece. The oximeter will faithfully reproduce saturation values over a wide range of thickness of the whole blood column (Fig. 2) but, following any change of light path distance, new standardization of the bias circuit with fully oxygenated blood is required. A portion of the unknown blood sample, collected in an oiled heparin syringe, is saved in a fluoride tube for Van Slyke manometric analysis. The last 2 ml of blood contained in the syringe are passed into the cuvette. This quantity of blood is sufficient to fill the cuvette allowing 0.5 ml to flush the lighted segments of plastic tubing. Both infra-red and bias circuit galvanometer readings are made. The infra-red deflection records changes in total hemoglobin concentration. The bias circuit reading indicates the oxygen saturation. Constancy of the infra-red galvanometer reading for successive blood samples indicates absence of air or oil bubbles in the light path and constancy of hemoglobin concentration. The entire length of the plastic tubing can be inspected for trapped air or oil at any time during the procedure. Usual room lighting produces no significant error in saturation determinations. Between readings the cuvette is left filled with blood being flushed with isotonic sodium chloride solution and air just prior to introduction of the next sample. This maneuver

† Coleman Anoxia Photometer Model 17A, Coleman Instrument Company, Maywood, Ill.

[2] Millikan, G. A., *Rev. Sci. Inst.*, 1942, **13**, 434.

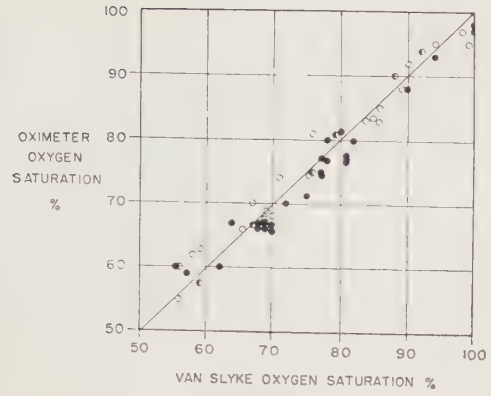


FIG. 3.

Comparison of oxygen saturation determinations on 54 blood samples, as determined by the oximeter and by the Van Slyke manometric method. Black points represent polythemic blood samples having an oxygen capacity from 29.1 to 33.2 volumes %. Half-black circles represent anemic blood samples having an oxygen capacity of 9.0 volumes %. Circles represent blood samples of intermediate hemoglobin concentration (14.0 volumes % to 23.0 volumes % oxygen capacity).

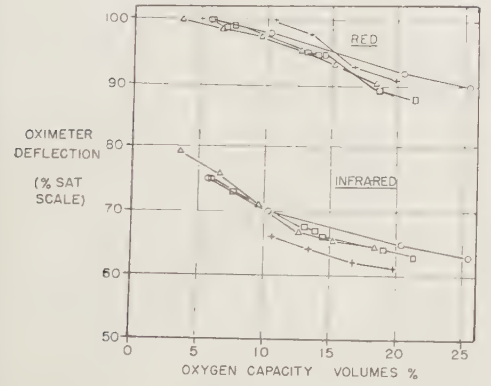


FIG. 4.

Variations of apparent oxygen saturation produced by change of hemoglobin concentration after standardization of the oximeter circuits. Fully saturated blood was diluted serially with plasma. The most dilute blood sample was used to set the bias circuit (RED) at 100% saturation, and, without change of galvanometer settings, the fully saturated samples of higher oxygen capacity measured. Although the true oxygen saturation of all samples was 100%, there was a fall in oxygen saturation as measured by the oximeter with more concentrated blood specimens, indicating incomplete compensation for changes of hemoglobin concentration.

minimizes exhaustion of the barrier layer photocells.

*Results.* Comparison of blood oxygen saturation determinations by the oximeter and by the manometric method of Van Slyke and Neill[3] is shown in Fig. 3. The standard error between observations by the two methods on 54 blood samples was 2.4% oxygen saturation. Determinations were made using human blood samples taken at the time of right heart catheterization and samples of dog blood desaturated by tonometric equilibration with nitrogen and air. The oxygen capacity of these samples varied from 9.0 volumes % to 33.2 volumes % without affecting accuracy significantly. The effect of wide variations of hemoglobin concentration following standardization is shown in Fig. 4. Blood from stock dogs was serially diluted with dog plasma and equilibrated with air at room temperature. The most dilute blood-plasma mixture was used to set the bias circuit at 100% saturation. The apparent oxygen saturation reading and infrared galvanometer reading were then recorded for the more concentrated fully saturated blood-plasma mixtures. The observations during four such experiments are plotted against oxygen capacity in Fig. 4. A change of oxygen capacity on the order of 10 to 20 volumes % is required to produce a change of 10% in the observed oxygen saturation. Thus although the bias circuit does not completely compensate for wide fluctuations in hemoglobin concentration, any slight variation of hemoglobin concentration due to fluid infusion and serial sampling during catheterization should not affect saturation readings appreciably.

If it can be shown that the light transmission of the empty cuvette remains constant, modification of this recording circuit would permit measurement of the entire length of galvanometer deflection on introduction of blood into the cuvette and consequently would allow direct reading of oxygen saturation without the step of standardization with oxygen-saturated blood. However, the assumption of constancy of light transmission is not warranted since after several hours' use there is gradual fogging of the plastic tube exposed to the 6 v earpiece lamp. After several hours' use, the plastic tubing is replaced.

[3] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

Standardization with oxygenated blood prior to each period of use eliminates this source of error. In addition the introduction of a more sensitive galvanometer would reduce the portability of the apparatus.

Agreement within 4.8% saturation between oximeter and manometric values can be expected ninety-five percent of the time. However, we have not used the oximeter other than as a scouting device at the time of catheterization checking all results with Van Slyke analyses prior to calculation of flow components.

*Summary.* (1) A polythene tubing cuvette is described which adapts the Millikan oximeter for measurement of oxygen saturation of drawn samples of whole blood.

(2) Oxygen saturation values agree with Van Slyke manometric values with a standard error of 2.4%.

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## Intravenous Amino Acid Tolerance Studies in Humans.* (17372)

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The introduction of specific and sensitive microbiological methods for the assay of amino acids has made possible the further study of many fundamental problems in the metabolism of these nutrients. The increasing clinical use of amino acids in parenteral nutrition, particularly in surgery, further emphasizes the necessity for more detailed studies on the fate of these substances in the human organism. For this purpose the rates of disappearance of intravenously administered amino acids have been investigated. The problem was first studied with *DL*-methionine as a test amino acid.[1-3] In view of the recent work on the importance of the simultaneous presence of all of the essential amino acids if maximal nitrogen retention is to occur,[4] it was felt that further tolerance studies should be conducted with a mixture of the 10 amino acids originally thought to be indispensable to man. The present paper reports the results of such studies on normal human males in intravenous tolerance to 10 amino acids when injected simultaneously. Included also are the data on the urinary excretion of the injected amino acids.

*Experimental.* The amino acid preparation used was the VUJ-N mixture kindly supplied by Merck & Company. The product contained varying amounts of the 10 indispensable amino acids plus additional glycine. After a 12-hour fast, blood samples were withdrawn for determination of fasting levels of the 10 amino acids. The amino acid mixture was then injected intravenously as rapidly as possible using 50 ml syringes (about 4-5 minutes) at a level of 1 ml per pound body weight. Blood samples were taken at 15, 30, 60, 120 and 180 minutes after the injection, and urine excreted during the 2-hour period immediately prior to the injection as well as during the period when the bloods were taken was also collected for assay. Ten amino acids were then determined by microbiological methods on heat deproteinized plasma from heparinized blood, and on the urines. The basal medium used was either that of Henderson and Snell,[5] or Steele, Sauberlich, Rey-

* Presented before the Society of University Surgeons, Tenth Annual Meeting, San Francisco, March 24, 1949.

1 Harper, H. A., Kinsell, L. W., and Barton, H. C., *Science*, 1947, **106**, 309.

2 Kinsell, L. W., Harper, H. A., Barton, H. C., Michaels, G. D., and Weiss, H. A., *Science*, 1947, **106**, 589.

3 Kinsell, L. W., Harper, H. A., Barton, H. C., Hutchin, M. E., and Hess, J. R., *J. Clin. Invest.*, 1948, **27**, 677.

4 Geiger, E., *J. Nutr.*, 1948, **34**, 97.

5 Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

TABLE I.
Plasma and Urine Amino Acid Levels Prior and Subsequent to Injection of 131 ml of a Mixture of 10 Amino Acids (VUJ-N).
Subject, K.H. ♂ 131 Lbs.

| Amino acid inj. (mg L isomer) | Leucine 1640 | Lysine 1390 | Isoleucine 1073 | Valine 753 | Histidine 524 | Methionine 524 | Phenylalanine 341 | Threonine 245 | Arginine 219 | Tryptophan 30 |
|---|---|---|---|---|---|---|---|---|---|---|
| Time (min.) | | | | Blood plasma levels (mg per 100 ml) | | | | | | |
| 0 | 1.6 | 3.2 | 0.8 | 3.7 | 2.1 | 0.2 | 1.3 | 1.6 | 1.3 | 1.3 |
| 15 | 6.2 | 7.8 | 4.5 | 10.4 | 3.2 | 2.0 | 1.8 | 2.4 | 3.5 | 1.8 |
| 30 | 4.7 | 5.8 | 2.4 | 6.7 | 2.9 | 1.2 | 1.7 | 2.2 | 2.0 | 1.7 |
| 60 | 2.9 | 4.2 | 1.5 | 4.9 | 2.4 | 1.0 | 1.6 | 1.7 | 1.2 | 1.4 |
| 120 | 2.2 | 3.4 | 0.9 | 3.8 | 2.0 | 0.4 | 1.4 | 1.4 | 1.2 | 1.2 |
| 180 | 2.1 | 3.0 | 0.9 | 3.7 | 1.8 | 0.3 | 1.3 | 1.2 | 1.2 | 0.9 |
| | | | | Urine levels (μg per hr) | | | | | | |
| 2 hr prior to inj. | 78 | 200 | 24 | 104 | 4000 | 24 | 64 | 320 | 440 | 310 |
| 3 hr post inj. | 363 | 2406 | 91 | 182 | 13600 | 182 | 647 | 1816 | 1020 | 363 |
| Diff. | 285 | 2206 | 67 | 78 | 9600 | 158 | 583 | 1496 | 580 | 53 |
| % of inj. dose exer. in 3 hr | 0.05 | 0.48 | 0.02 | 0.03 | 5.50 | 0.09 | 0.51 | 1.83 | 0.79 | 0.53 |

nolds, and Baumann.[6] *Leuconostoc mesenteroides,* P-60 was employed as the assay organism for all amino acids except threonine and arginine for which *Streptococcus fecalis* was preferred.

*Results and discussion.* In Table I there are listed the absolute amino acid levels (*L*-isomers, mg per 100 ml plasma) which were observed before and at various intervals after the amino acid mixture was infused in a normal male subject chosen as typical of 5 experiments. This table also lists the quantities of the 10 amino acids (μg per hour) excreted during the 2-hour period preceding the infusion as well as for the 3-hour period during which blood samples were taken. From this data the percentage of the injected dose which was excreted during the period of observation was calculated.

The amino acid pattern infused is also compared (Fig. 1) to that obtaining in the plasma of 2 subjects at various intervals subsequent to the injection. For this purpose each amino acid is first corrected for its fasting level and all concentrations are then expressed as a percentage of the total amino nitrogen measured in the form of the 10 amino acids. In order to compare the excreted with the infused pattern the individual contribution (% amino nitrogen) of each amino acid to the total 3-hour amino nitrogen excretion of the 10 amino acids is also shown in Fig. 1.

It will be noted that while the amino acid levels at 15 minutes after the injection are more or less elevated in proportion to the amount injected, subsequently the levels fall rapidly, in some instances decreasing to substantially lower than the fasting levels. It is possible that this latter effect is referable to a synthetic process which requires some of the original amino acid supply of the body. The comparison of the pattern of 10 amino acids in the plasma at various intervals after the injection with that originally infused reveals some differences between the two subjects in the rate of disappearance of individual acids. In Subject K.H., the preponderance of leucine, valine, and methionine is noteworthy.

[6] Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.,* 1949, **177,** 533.

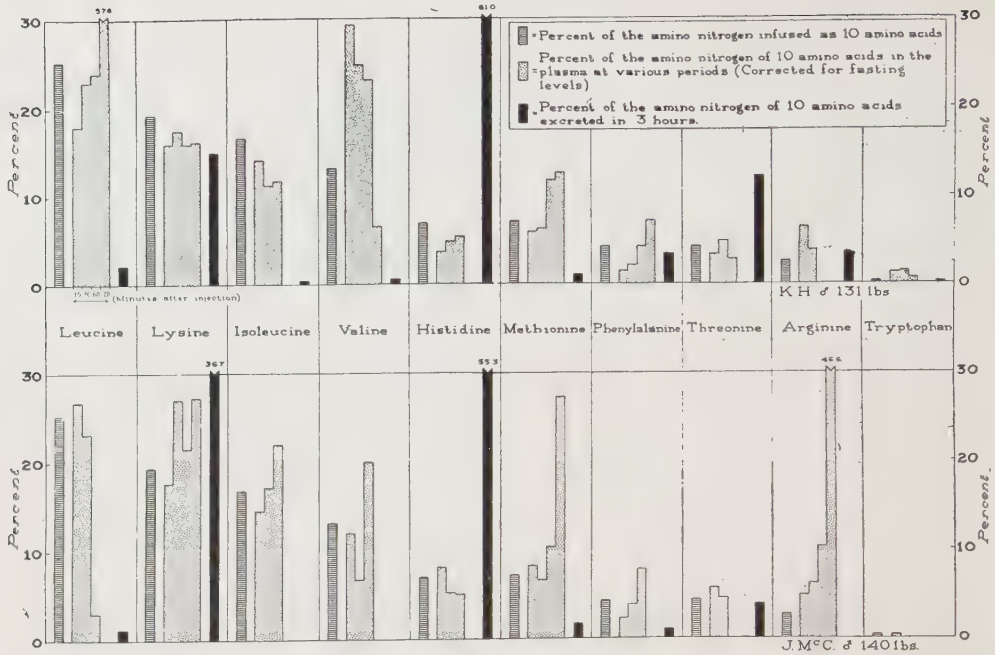


FIG. 1.

Comparison of amino acid pattern infused with that of the plasma at intervals subsequent to injection. The individual contribution of each amino acid to the total 3-hour amino nitrogen excretion of 10 amino acids is also shown. All concentrations are expressed as a percentage of the total amino nitrogen measured in the form of 10 amino acids.

In Subject J. McC., lysine, methionine, and arginine are responsible for the excess over the infusion mixture, in the later periods. Only the persistence of methionine is common to both subjects. The other acids retain a close resemblance to the infusion mixture during most of the period of observation. The effect of a change in the concentration of any one amino acid on the uptake of the others would be of interest. This experiment is planned as an extension of the basic tolerance studies.

The pattern of amino acids excreted bears no relationship to that infused. Very little quantitative data on the renal threshold for amino acid excretion in humans is available. One fact that is well established, however, is that the reabsorptive capacity of the kidney for the natural *L* isomers, in contrast to the *D* isomers, is relatively high. At the infusion levels used in these experiments, histidine, lysine and threonine accounted for the majority of the urinary amino nitrogen measured. One could therefore disregard the losses by the renal route of the other amino acids.

Silber and his collaborators[7] have infused into dogs an amino acid mixture similar to that used in our experiments and studied the pattern of the essential amino acids excreted in the urine. They also point out that the urine pattern did not resemble the pattern infused nor was it greatly altered by protein depletion. However, in a subsequent paper, Silber[8] by infusing 3 different amino acid mixtures in dogs was able to show a relationship between intake and excretion and suggested that this approach might be useful in designing amino acid mixtures for specific types of patients. From our experiments it would seem that because of the high renal threshold for most amino acids, the urine fails adequately to reflect either blood levels or extent of uptake by the tissues. In liver disease we have repeatedly observed plasma levels of *L*-methionine, for example, which far

7 Silber, R. H., Seeler, A. O., and Howe, E. E., *J. Biol. Chem.*, 1946, **164**, 639.

8 Silber, R. H., Howe, E. E., and Porter, C. C., *Trans. N.Y. Acad. Sci.*, 1948, Ser. II, **10**, 277.

exceeded normal without any resulting marked increase in excretion. In the present experiments, the rate of disappearance of leucine from the blood is much greater than that of histidine but these differences are not reflected in the urine. It is therefore felt that studies of amino acid changes in the blood plasma may be more valid for the purpose of an investigation of utilization of various mixtures.

A comparison of tolerance to the same quantities of amino acids when infused at a constant rate over a one-hour period is now in progress. The results will be reported in a later communication.

*Summary.* A mixture of the 10 indispensable amino acids has been injected intravenously in normal human male subjects and the plasma levels of these 10 acids as well as the urinary excretion, prior and at various intervals subsequent to the injection, were determined microbiologically. The pattern of amino acids excreted is shown to bear no relationship to that infused.

The work here reported has been carried out with the active cooperation of Drs. H. J. McCorkle, of the University of California Medical School, and H. L. Silvani, Frank Choy, and their associates at The Veterans' Hospital, Fort Miley.

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## Intravenous Administration of Massive Dosages of Estrogen to the Human Subject; Blood Levels Attained. (17373)

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The employment of estrogens in the management of cases of cancer of the breast[1] and prostate[2] necessitates our further knowledge of the biological effects of the estrogens when administered in varying dosage and by various routes.

Few data are available concerning the effects of intravenous administration of estrogen in the human subject. Loeser[3] injected as much as 10 mg estrone, 25 mg estradiol, or 150 mg stilbestrol intravenously in propylene glycol and observed an elevation of intrauterine temperature presumably attributable to increased uterine blood-flow. He noted no toxic side reactions and indicated that the medication was well tolerated. Abarbanel[4] reported the administration of small doses of estrone sulfate for the control of uterine bleeding.

We wish to report on the clinical tolerance of intravenous infusions of massive dosages of an aqueous solution of conjugated natural estrogens.* We have also determined by bioassay the blood levels attained immediately following infusion and at fixed intervals after treatment.

*Materials and methods.* Thirteen female and 9 male subjects have been studied. Their age, race, and clinical diagnosis are indicated in Table I. All patients were at bed rest when treated. Intravenous infusions in the dose and volume indicated were carried out in the customary manner employing the antecubital vein. A buffered aqueous solution of conjugated estrogens in the form of a concentrate from pregnant mare's urine was mixed with normal saline so that a final concentration of from 0.5 to 2 mg equivalents of estrone sulfate per cc was obtained for injection. The rate of flow was so adjusted that the total dose was administered in from 35 to 60 minutes. Each patient's temperature, pulse,

1 Nathanson, I. T., *Surg. Clin. N. A.*, 1947, **27**, 1144.

2 Huggins, C., Stevens, R. E., and Hodges, C. V., *Arch. Surg.*, 1941, **43**, 209.

3 Loeser, A. S., *J. Obst. and Gynec. Brit. Emp.*, 1948, **55**, 17.

4 Abarbanel, A. R., paper No. 71; read by title; Assn. for Study of Internal Secretions, June, 1949.

* The preparation used was "Premarin" (injectable) kindly supplied by the Ayerst, McKenna & Harrison Ltd., through the courtesy of Drs. G. H. C. McKeown and E. C. Reifenstein, Jr.

TABLE I. Intravenous Estrogen Administration.

| Patient | Sex and color | Age | Date treated | Total dose (mg) | Serum level of estrogens (μg) | | | | | | | Reaction | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Pre. | Post | 2 hr | 4 hr | 6 hr | 8 hr | 24 hr | | |
| E.T. | C.F. | 62 | 2/16/49 | 200* | .0 | 36 | 8.0 | 3.2 | | | | 0 | Cancer of breast, with wide local extension; arteriosclerotic degeneration of the brain |
| | | | 18 | 200* | .0 | 24.4 | 12.0 | 3.2 | | | | 0 | |
| | | | 3/ 4 | 200* | | 38 | | | | .0 | | 0 | |
| | | | 9 | 200* | .0 | 30 | 5.6 | .0 | .0 | .0 | .0 | 0 | |
| | | | 15 | 300* | .0 | 35 | 7.6 | 4.0 | .0 | .0 | .0 | 0 | |
| | | | 18 | 300* | .0 | 39 | 4.3 | 2.4 | | .0 | .0 | 0 | |
| | | | 5/17 | 100† | .0 | 23.2 | | | | .0 | .0 | 0 | |
| | | | 25 | 300* | .0 | 37 | | 5.6 | | .0 | .0 | 0 | |
| | | | 27 | 400* | .0 | 35 | 18.4 | 8.6 | 6 | 3.8 | | 0 | |
| | | | 31 | 400* | .0 | 38 | 16.0 | 7.0 | | .0 | .0 | 0 | |
| N.H. | C.F. | 76 | 2/16 | 200* | .0 | 21.8 | | | | | | 0 | Cancer of breast, pulmonary and osseous metastases; hypertensive heart disease |
| | | | 18 | 200* | .0 | 22.4 | | | | | | 0 | |
| | | | 3/ 3 | 400* | | | | | | | | (c) | |
| R.A. | W.F. | 53 | 3/ 4 | 200* | .0 | 28.4 | 5.6 | 5.4 | 2.4 | .0 | | 0 | Bilateral, inoperable breast cancer with metastases |
| | | | 9 | 200* | .0 | 19.0 | | | 2.2 | .0 | .0 | 0 | |
| | | | 15 | 300* | .0 | 23.8 | | 3.6 | | .0 | .0 | 0 | |
| | | | 18 | 300* | .0 | 39 | 5.6 | 3.2 | 3.2 | .0 | .0 | 0 | |
| J.F. | C.M. | 55 | 3/17 | 200* | .0 | 24.4 | 7.2 | | | | .0 | 0 | Carcinoma of prostate; osseous metastases; moribund |
| | | | 21 | 200* | .0 | 25.8 | 6.0 | | | 7.4 | .0 | 0 | |
| J.S. | W.M. | 67 | 3/17 | 200* | .0 | 23.8 | 7.0 | | | | | 0 | Carcinoma of prostate |
| L.H. | W.F. | 73 | 3/25 | 200* | .0 | 22.4 | 3.6 | .0 | .0 | .0 | | 0 | Massive, fungating breast cancer; severe anemia |
| | | | 28 | 240* | .0 | 21.2 | 2.0 | .0 | .0 | .0 | .0 | 0 | |
| | | | 31 | 200* | | 17.6 | | | .0 | | .0 | 0 | |
| | | | 5/ 5 | 200* | .0 | 25.6 | | 2.4 | | .0 | .0 | 0 | |
| | | | 10 | 250* | .0 | 21.2 | | 2.0 | | .0 | .0 | 0 | |
| J.W. | W.F. | 41 | 3/28 | 100† | .0 | 22.4 | .0 | .0 | .0 | | .0 | 0 | Cancer of breast; pulmonary metastases with pleural effusion |
| | | | 30 | 132‡ | .0 | 22.0 | 3.2 | | .0 | .0 | .0 | 0 | |
| C.D. | W.M. | 65 | 4/ 4 | 200* | .0 | 24.0 | 10.0 | | 7.6 | | | 0 | Cancer of prostate, huge abdominal masses |
| L.S. | W.M. | 59 | 4/ 4 | 200* | .0 | 21.6 | 6.0 | | .0 | | | 0 | Cancer of prostate |
| A.T. | W.M. | 68 | 4/ 8 | 200* | 3.2 | 25.6 | 9.6 | .0 | | .0 | | 0 | Carcinoma of prostate with metastases to bone and lymph node |
| P.C. | C.M. | 81 | 4/11 | 200* | .0 | 23.6 | 8.4 | 4.2 | | .0 | | 0 | Carcinoma of prostate |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C.M. | W.M. | 63 | 4/15 | 200* | .0 | 28.4 | 7.6 | 3.2 | | .0 | .0 | 0 | Cancer of prostate with metastases |
| | | | 22 | 200* | | 26.4 | 8.4 | 2.4 | | .0 | .0 | 0 | |
| | | | 24 | 200* | .0 | 23.8 | | | | | .0 | 0 | |
| | | | 26 | 200* | .0 | 21.0 | 6.8 | | 2.8 | | .0 | 0 | |
| | | | 28 | 200* | .0 | 24.4 | | 6.4 | | 2.4 | .0 | 0 | |
| | | | 5/ 3 | 200* | .0 | 26.4 | 4.0 | | | 3.8 | | 0 | |
| | | | 5 | 250* | .0 | 25.6 | | 6.8 | | 2.8 | | 0 | |
| M.W. | C.F. | 58 | 5/10 | 200* | .0 | 22.4 | 6.8 | 3.6 | | .0 | .0 | 0 | Massive, fungating breast cancer; marked debility |
| | | | 17 | 200* | .0 | 21.2 | 7.6 | 4.8 | | 5.0 | .0 | 0 | |
| | | | 25 | 300* | .0 | 33.0 | 14.0 | 8.4 | 4.0 | 7.2 | .0 | 0 | |
| L.J. | C.F. | 79 | 5/25 | 200* | .0 | 23.8 | 6.0 | .0 | | .0 | .0 | 0 | Advanced breast cancer; marked senile degeneration |
| | | | 27 | 300* | .0 | 38.0 | 12.8 | | 2.6 | 2.0 | .0 | 0 | |
| | | | 31 | 400* | .0 | 38 | 14.4 | 7.6 | | 3.4 | .0 | 0 | |
| A.F. | W.F. | 45 | 6/ 2 | 300* | .0 | 31 | .0 | .0 | | .0 | .0 | 0 | Breast cancer with osseous metastases |
| | | | 9 | 400* | .0 | 32 | 13.2 | 3.2 | | .0 | | | |
| M.M. | C.F. | 71 | 6/ 2 | 300* | .0 | 35 | 13.6 | 6.8 | | 3.4 | .0 | 0 | Breast cancer with osseous metastases |
| | | | 9 | 300* | .0 | 45 | 14.8 | | | | | (a) | |
| J.F. | C.M. | 66 | 6/ 9 | 175§ | .0 | 35 | 3.6 | | | | .0 | 0 | Cancer of prostate |
| C.P. | W.F. | 73 | 6/10 | 250* | .0 | 35 | 10.0 | 4.2 | | .0 | .0 | 0 | Severe rheumatoid arthritis |
| | | | 14 | 300* | .0 | 39.8 | 14.4 | 7.6 | | 2.8 | .0 | 0 | |
| | | | 15 | 300* | .0 | 33 | | | | | | 0 | |
| | | | 16 | 300* | .0 | 32 | | | | | | 0 | |
| | | | 17 | 300* | .0 | 37 | | | | | | 0 | |
| F.J. | C.M. | 44 | 6/10 | 250* | .0 | 38 | 14.0 | 6.0 | | 2.0 | .0 | 0 | Severe rheumatoid arthritis |
| | | | 14 | 300* | .0 | 36 | 12.8 | 5.6 | | 2.0 | | 0 | |
| | | | 15 | 300* | .0 | 39.8 | | | | | | 0 | |
| | | | 16 | 300* | .0 | 34 | | | | | | 0 | |
| | | | 17 | 300* | .0 | 35 | | | | | | 0 | |
| J.D. | C.F. | 43 | 6/10 | 250* | .0 | 39 | 12.8 | 3.2 | | | | 0 | Severe rheumatoid arthritis |
| | | | 14 | 300* | .0 | 32 | 13.2 | 6.6 | | | | 0 | |
| | | | 15 | 300* | .0 | 48 | | | | | | (b) | |
| | | | 16 | 300* | .0 | 35 | | | | | | 0 | |
| | | | 17 | 300* | .0 | 35 | | | | | | 0 | |
| D.M. | C.F. | 59 | 6/16 | 207‖ | .0 | 19 | | 1.5 | | .0 | | 0 | Advanced cervical cancer; frozen pelvis |
| F.E. | W.F. | 68 | 6/15 | 250* | .0 | 45 | 14.8 | 8.0 | | 6.0 | | 0 | |
| | | | 16 | 225* | .0 | 38 | | | | 7.8 | | 0 | |
| | | | 17 | 300* | 5.8 | 43 | | 18.0 | | 7.8 | | 0 | |

* Given in 200 cc normal saline.
† " " 100 " " "
‡ " " 132 " " "
§ " " 175 " " "
‖ " " 165 " " "

(a) Mild chill; infusion discontinued.
(b) Nausea for 6-8 hours after infusion.
(c) Died following infusion—see text.
Values less than 1 $\mu$g doubtful and indicated by 0.0.

respiration and blood pressure were recorded before, during and after the infusion. Blood samples were drawn for estrogen bioassay just prior to the beginning of the infusion, at the conclusion of the injection, and at stated intervals throughout the subsequent 8 to 24 hours. The drawn blood was permitted to clot and the serum promptly prepared.

The estrogen content of the sera was determined by a modification of the uterine weight method of Lauson *et al.*[5] Twenty-one day-old female rats of the N.I.H. or Holtzman strain were ovariectomized and were given the proper dilution of serum in 0.25 cc twice daily for 2 days. Twenty hours after the last injection the test animals and uninjected controls were autopsied, the uterus dissected out, freed of fluid and weighed to the nearest milligram on a Roller-Smith torsion balance. The estrogen content of the patients' sera was calculated on the basis of the activity of an aqueous dilution of the same preparation of conjugated estrogens that had been employed for the clinical studies. Duplicate and multiple determinations at various levels have indicated an approximate error of 10% in our estrogen bioassays.

*Results and discussion.* The clinical reaction of 67 infusions of from 100 to 400 mg each was observed. All but 3 infusions were attended by no subjective reactions on the part of the patient. One patient (J.D.) who had received 200 mg complained of nausea for 6 hours following the injection. Another patient (M.M.) who had received 350 mg suffered a mild chill necessitating the discontinuation of the infusion. The third patient (N.H.) who was in a badly debilitated state from advanced metastatic breast cancer and hypertensive heart disease died shortly after the administration of 400 mg of conjugated estrogens. The terminal clinical picture simulated that of cerebrovascular accident. Careful autopsy examination, including study of the brain, revealed no apparent basis for this patient's death. In view of her general debility and the total lack of reaction on the part of other patients to identical treatment, it may be considered that her death was not causally related to the estrogen administration.

The pulse, temperature, blood pressure and respiration in the remaining cases were not significantly affected. The apprehension attendant upon the venipuncture induced a slight but transient rise of blood pressure in some cases, particularly upon the first infusion. This momentary effect subsided rapidly and in most cases was not observed during subsequent infusions. Hyperpyrexia was observed only in the one patient (M.M.) who had experienced a reactive chill.

On the whole, the infusions were remarkably well tolerated in spite of the very high doses employed. Moreover, it should be noted that most of our subjects were all bedridden, debilitated individuals with advanced cancer of the breast or prostate and even greater tolerance might be anticipated from patients in better general clinical condition.

The blood levels of estrogen attained immediately following the infusion varied from 17.6 to 48 $\mu$g per cc and rapidly dropped off to negligible levels in most cases after 8 hours. This indicates a fairly ready dilution of the estrogen in the blood and a rapid removal therefrom. Studies on the urinary excretion and metabolic fate of the injected estrogen will be reported subsequently.

These preliminary observations indicate the feasibility of procuring an inordinately high blood level of estrogen in a short time. The quantitative data will permit an estimate of the rate of estrogen infusion which may be required for the maintenance of such levels for longer periods of time. The potential usefulness of such intensive estrogenization in patients with prostatic and breast cancer remains to be determined. We have noted no material clinical effect on the malignant process from the limited number of infusions thus far administered. The evaluation of such potential effects will require more prolonged periods of sustained treatment and observation.

*Summary.* Thirteen female and 9 male subjects have been given a total of 67 intravenous infusions of from 100 to 400 mg of conjugated natural estrogens dissolved in 200 cc of normal saline. In the main, these in-

[5] Lauson, H. D., Heller, C. G., Golden, J. B., Severinghaus, E. L., *Endocrinology,* 1939, **24**, 35.

fusions were well tolerated. Serum levels of estrogen as determined by bioassay varying from 17.6 to 48 μg were obtained and only negligible quantities of estrogen remained in the blood after 8 hours.

The feasibility of rapidly inducing a high serum estrogen level by intravenous infusion is demonstrated. The clinical usefulness of this form of estrogen therapy in carcinoma of the breast and prostate remains to be evaluated.

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## Blood Pressure in the Rat.* (17374)

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The work of Kersten *et al.* on the indirect measurement of blood pressure in the rat[1,2] will increase the use of rats for studying various phases of the hypertension problem. If rats of different ages are to be used it should be remembered that "a tendency to a slight rise in blood pressure with age" has been noted.[3] It was the scope of the following investigations to establish accurate data which the literature does not provide.

Rats of the Long-Evans strain were kept on Friskies and water. The blood pressure was measured in unanesthetized, unheated animals with the foot method described by Kersten, Brosene, Ablondi and SubbaRow.[1] Readings obtained about 1 minute apart in the same animal rarely differed more than ± 3 mm Hg.

Fig. 1 shows that the systolic blood pressure increases with age in the growing rat. Within the first 2 months up to a weight of 150 g the pressure increases rapidly. A slower, but still progressive rise is noted in rats weighing 200 to 350 g.

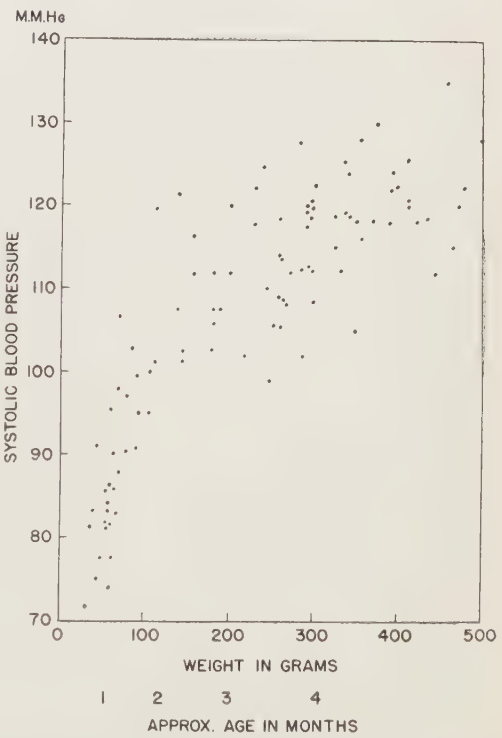


FIG. 1.

Systolic blood pressure in rats of different weights and ages.

The age conditioned differences in the sys-

* Aided by a grant from the Life Insurance Medical Research Fund.

1 Kersten, H., Brosene, W. G., Jr., Ablondi, F., and SubbaRow, Y., *J. Lab. and Clin. Med.*, 1947, **32**, 1090.

2 Ablondi, F., SubbaRow, Y., Lipchuck, L., and Personens, G., *J. Lab. and Clin. Med.*, 1947, **32**, 1099.

3 Griffin, John A., and Parris, E. J., The Rat in Laboratory Investigation, J. B. Lippincott Co., Philadelphia-Montreal-London, 1942, p. 286.

† An apparatus was obtained from the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of the late Dr. Y. SubbaRow.

tolic blood pressure in the rat are of a magnitude which deserves attention. A pressure of 120 mm Hg, normal for a rat weighing 300 to 500 g, is definitely hypertensive for a rat weighing less than 100 g.

The blood pressure in man increases in the corresponding age groups of infancy and early childhood in a similar fashion.[4,5] The consideration of this fact is common practice in pediatric experience.

*Summary.* The systolic blood pressure has been recorded in 100 unanesthetized, unheated rats of various ages. It increases with weight and age and normal values were established.

[4] Graham, Archibald W., Hines, Edgar A., Jr., and Gage, Robert P., *Am. J. Dis. Child.*, 1945, **69**, 203.

[5] Downing, Elizabeth M., *Am. J. Dis. Child.*, 1947, **73**, 293.

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## Budding of Thrombocytes from Megakaryocytes.* (17375)

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It is now generally agreed that the megakaryocyte is the source of the thrombocyte.[1] Dameshek and Miller[2] discuss the changes that occur in the marrow in idiopathic thrombocytopenic purpura. The latter authors found that, in the marrows of 10 normal subjects, an average of 68.6% of the megakaryocytes showed thrombocyte budding. Cartwright and associates[3] found thrombocyte budding in 75% of megakaryocytes in normal marrows. In a recent study of bone marrow films made by the citrate technic, as described below, from 12 patients with idiopathic thrombocytopenic purpura before operation, it was found that 0.14% of the megakaryocytes showed evidence of thrombocyte budding. Eighteen postoperative films, made at varying intervals after operation on 10 of these patients showed thrombocyte budding from 9.2% of megakaryocytes. When the bone marrows of 7 normal control patients without hematological abnormality were examined, however, thrombocyte budding was seen in only 0.43% of the megakaryocytes. This discrepancy in the results obtained in the normal suggested that the differences might be due to variations in the technic used. Since the routine method for making bone marrow films in this Institute involves the use of a dry syringe, further investigations were carried out on material obtained here in this way.

*Methods. Citrate method.* The puncture of the sternum was performed with a Salah needle introduced at the level of the third or fourth intercostal space. The syringe and needle were first washed out with sterile 3.8% solution of sodium citrate, and aspirated material, usually 1 cc or less, was expressed on a watch glass which also had been rinsed with sodium citrate. Flecks of marrow were gently lifted with tweezers, and placed on carefully cleaned glass slides. They were then spread by means of another glass slide which was laid on top of the marrow flecks and slid rapidly across the surface of the first slide. The marrow films were dried in air. In the investigations referred to above, May-Grunwald-Giemsa stain had been used. In the investigations described below, Wright's stain was used, and the technic differed in that, following aspiration with a dry syringe, a sec-

* The author is glad to acknowledge the assistance of Dr. C. C. Sturgis and Dr. F. H. Bethell in providing the facilities for the carrying out of this study.

† Rockefeller Travelling Research Fellow.

[1] Tocantins, L. M., *Medicine*, 1938, **17**, 155.

[2] Dameshek, W., and Miller, E. B., *Blood*, 1946, **1**, 27.

[3] Cartwright, G. E., Chung, Hui-Lan, and Chang, An, *Blood*, 1948, **3**, 249.

TABLE I.
Ten Normal Marrows Studied by the Dry Technic.

| | Fields with megakaryocytes | | Fields without megakaryocytes | | |
|---|---|---|---|---|---|
| | Thrombocytes per 100 megakaryocytes | Clumps of thrombocytes per 100 meg. | Thrombocytes per 100 fields | Clumps of thrombocytes per 100 fields | % of meg. with thrombocytes |
| Areas with structure | 387 | 16 | 369 | 8 | 25 |
| Areas with no structure | 1496 | 24 | 887 | 8 | 65 |

TABLE II.
Four Cases of Idiopathic Thrombocytopenic Purpura Studied by the Dry Technic.

| | Fields with megakaryocytes | | Fields without megakaryocytes | | |
|---|---|---|---|---|---|
| | Thrombocytes per 100 megakaryocytes | Clumps of thrombocytes per 100 meg. | Thrombocytes per 100 fields | Clumps of thrombocytes per 100 fields | % of meg. with thrombocytes |
| | | Presplenectomy | | | |
| Areas with structure | nil | nil | nil | nil | nil |
| Areas with no structure | 9 | nil | 7 | nil | nil |
| | | Postsplenectomy | | | |
| Areas with structure | 36 | nil | 13 | nil | 2 |
| Areas with no structure | 1117 | 12 | 475 | nil | 59 |

ond syringe that had been washed out with sodium citrate was used to aspirate a second sample of marrow, the further steps being those just described.

*Dry method.* The puncture was performed as before. A perfectly dry needle was used and a dry syringe attached to it. A sample of marrow, usually 1 cc or less, was rapidly aspirated. A small amount was then quickly expressed through another needle on to a clean cover slip. Excess fluid was withdrawn by means of the syringe, and the film was spread by means of a second cover slip. The films were stained with Wright's stain, and mounted on glass slides.

Ten normal marrow films made by the dry technic were first studied. Regardless of the technic used, it is common to find areas in which there is some preservation of marrow structure; fat spaces may be seen in many instances, although at other times there is merely a large aggregation of cells which can be observed with the naked eye. There are also other areas in which the cells lie quite free as in a blood film. The percentage of megakaryocytes that showed budding in areas with marrow structure and in areas without such structure were estimated separately. In addition, each time a megakaryocyte was encountered, another field 3 oil immersion fields distant from it was studied, and the number of thrombocytes contained therein was enumerated. Sometimes it was possible to count individual thrombocytes. At other times they had to be counted as clumps. In the normal marrows, a total of 100 megakaryocytes was examined in areas with marrow structure, and 100 in areas without such structure. In the other films it was possible to examine 25 megakaryocytes in each such area, and in some marrows, where budding was scanty, larger numbers of cells were used. Budding was recorded as being present when it appeared to be fairly definite, but this is a matter about which there may be divergence of opinion between different observers. The mere presence of megakaryocytes and thrombocytes in the same field was not considered to indicate true budding. The findings obtained by the dry technic in ten normal marrow speci-

TABLE III.
Five Patients Studied by Both the Dry and the Citrate Technics.

| | Fields with megakaryocytes | | Fields without megakaryocytes | | |
|---|---|---|---|---|---|
| | Thromb. per 100 megak. | Thromb. clumps per 100 meg | Thromb. per 100 fields | Thromb. clumps per 100 fields | % of megakaryocytes |
| Pernicious anemia (thrombocytes normal) | | | | | |
| Dry method | | | | | |
| Areas with structure | 248 | 4 | 556 | 4 | 20 |
| " " no structure | 748 | 32 | 476 | 24 | 52 |
| Citrate method | | | | | |
| Areas with structure | 120 | nil | 160 | nil | 15 |
| " " no structure | nil | nil | 8 | nil | 0 |
| Pernicious anemia (thrombocytes much reduced) | | | | | |
| Dry method | | | | | |
| Areas with structure | 64 | nil | 20 | nil | 8 |
| " " no structure | 332 | nil | 104 | nil | 60 |
| Citrate method | | | | | |
| Areas with structure | 12 | nil | 4 | nil | 2 |
| " " no structure | nil | nil | nil | nil | 0 |
| Chronic granulocytic leukemia (thrombocytes increased) | | | | | |
| Dry method | | | | | |
| Areas with structure | 168 | 60 | 312 | 28 | 52 |
| " " no structure | 1584 | 88 | 444 | nil | 92 |
| Citrate method | | | | | |
| Areas with structure | 404 | 44 | 320 | 20 | 48 |
| " " no structure | 76 | 12 | 56 | 4 | 16 |
| Chronic granulocytic leukemia (thrombocytes much increased) | | | | | |
| Dry method | | | | | |
| Areas with structure | 32 | 72 | 64 | 32 | 68 |
| " " no structure | 1696 | 212 | 1456 | 72 | 96 |
| Citrate method | | | | | |
| Areas with structure | 550 | 110 | 60 | 20 | 70 |
| " " no structure | 60 | 30 | 76 | 50 | 20 |
| Idiopathic thrombocytopenic purpura (platelets very much reduced) | | | | | |
| Dry method | | | | | |
| Areas with structure | 4 | nil | 12 | nil | 0 |
| " " no structure | 24 | nil | 4 | nil | 1 |
| Citrate method | | | | | |
| Areas with structure | nil | nil | nil | nil | 0 |
| " " no structure | nil | nil | nil | nil | 0 |

mens are shown on Table I.

Marrow aspirates obtained before and after splenectomy in four cases of idiopathic thrombocytopenic purpura were studied by the dry technic (Table II). In the peripheral blood, the thrombocytes were extremely few before operation. In 3 of the patients marrow films were made soon after splenectomy when the thrombocyte level was normal, and in the fourth case, the second puncture was made 3 years after operation at a time when the thrombocytes were recorded as being normal. Estimates of thrombocyte numbers rather than actual enumeration were employed because it is considered at this Institute that too much significance is apt to be attached to variations in the thrombocyte count which may, in fact, be due to technical factors or to differences in distribution of thrombocytes.

Sternal aspirations were performed on 5 patients with hematologic disorders and films were made by both dry and citrate methods. The results of the examination of the preparations are shown on Table III.

*Discussion.* There is obviously a very great difference between the results of thrombocyte counts on marrow aspirate obtained by the dry technic and that secured with the citrate method. It will be seen from Table III that there is less difference in areas where the marrow structure is preserved.

It would appear that when sodium citrate is used to flush out the syringe with which the sternal puncture is to be performed, many thrombocytes are removed from the marrow sample, and that this removal of thrombocytes is most marked in areas where the marrow structure is not intact. It might, therefore, be argued that the correct estimate of thrombocyte budding can be made only when a dry syringe is used. The figures given in Table III, however, indicate that even when this method is employed, the results are open to question, because here the estimates may be equally fallacious. When the thrombocyte count is high in the peripheral blood, one would expect that their number would also be increased in the marrow and in the blood which inevitably dilutes the marrow sample. The thrombocytes are sticky, and under the conditions of sternal aspiration one would expect them to adhere to one another and to the megakaryocytes, the largest cells which they encounter. Hence, it it would seem that counts performed with the dry technic are also inaccurate since much apparent thrombocyte budding is due to chance juxtaposition and adhesion of the sticky thrombocytes to the megakaryocyte. The estimate that is likely to be most valid is that made from areas with definite marrow structure. Even here, however, fields without megakaryocytes may contain an equal number of thrombocytes, and so it is not possible to say with certainty that in the normal marrow the number of megakaryocytes that show budding is as high as 25 per cent. Marrow structure is less well preserved when the citrate technic is used and it would appear that this method is unsatisfactory for investigations concerned with the budding of thrombocytes from megakaryocytes.

*Summary.* When marrow aspirations are performed using a syringe flushed with sodium citrate, a false impression is obtained of the amount of budding of thrombocytes from megakaryocytes because many thrombocytes are washed away from the parent cells.

When a dry technic is employed, the results are equally inaccurate because thrombocytes that by chance are in juxtaposition with megakaryocytes may stick to them and appear to be arising from them.

When aspirated marrow samples are used, the best indication of the extent of budding is obtained when a dry technic is used, and examination of the marrow is limited to areas of the film that have definite marrow structure. It is probable that less than twenty-five per cent of megakaryocytes in the normal marrow show true budding at any one time.

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# Administration of BAL* in Selenium Poisoning.† (17376)

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Selenium causes "alkali disease" or "blind staggers" in livestock in the Dakotas, Kansas, Montana, Wyoming, and other Western States where the soil and vegetation are high in selenium content. Therefore the toxic properties of selenium and its compounds are of great importance in these areas.

As far as we know, there is no reliable agent which is of value in preventing or curing the symptoms of selenium poisoning, though protective action of arsenic has been demonstrated by Moxon *et al.*[1]

Peters, Stocken, and Thompson[2] described the effectiveness of BAL in counteracting the effects of arsenical poisoning. The protective action of this compound in arsenical poisoning was believed to be due to the release of SH groups by BAL which could compete with the tissue SH groups for the arsenic, and act as a detoxifying agent. The nature of the reaction between arsenic and BAL made it seem probable that BAL would react similarly with other metals, and Gilman, Allen, and Philips[3] have reported its effectiveness as an antidote for bichloride of mercury and cadmium poisoning, and Braun, Lusky, and Calvery[4] showed similar results against poisoning by antimony, bismuth, chromium and nickel. However, they demonstrated that lead and selenium were made more toxic by BAL.

Our experiments performed using BAL as an antagonist against selenium poisoning confirmed the results already described.[4] However, we have found no explanation in the literature of the apparent synergism between BAL and selenium, hence our work has been directed along this line.

*Experimental.* Healthy white rats 6 to 8 weeks old, 120 and 150 g of weight were used. Selenium was administered by intraperitoneal injections in the form of a freshly prepared aqueous solution of sodium selenite (SS). Intramuscular injections of BAL freshly dissolved in peanut oil were used. Each drug was administered daily for varying lengths of time.

A. *Toxicity of selenium.* The toxicity of selenium was determined by injections of (SS) in daily doses of 5 mg/kg of body weight. (This is equivalent to 2.2 mg of the element selenium). The animals were maintained on this regimen for a period of 28 days at which time they were sacrificed and the livers were examined for any possible pathology. All rats survived the full experimental period. (Table I). Microscopic examination showed a typical picture of liver necrosis and fatty degeneration (Fig. 1) as previously described by other workers.[6] Microscopic examination of the kidney was negative.

B. *Toxicity of BAL.* In this group all rats survived for the experimental period after receiving BAL in doses of 15 mg/kg of body weight. (Table I). Liver and kidney showed no abnormalities.

C. *Toxicity of SS and BAL.* 1. Combined treatment with BAL and SS. Rats injected simultaneously with BAL and selenium, 15 mg/kg and 5 mg/kg, respectively, had an average survival period of 8 days ranging from 6 to 10 days; those receiving doses of 7.5 mg/kg BAL and 5 mg/kg of selenium had

* BAL was kindly supplied by Hynson, Westcott and Dunning, Inc., of Baltimore, Md., and the Army Medical Center, Bethesda, Md.

† A preliminary report was presented at the Federation of American Societies for Experimental Biology in Detroit, Mich., April 18-22, 1949.

1 Rhian, M., and Moxon, A. L., *J. Pharm. and Exp. Therap.*, 1943, **78**, 249.

2 Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616.

3 Gilman, A., Philips, F. S., Allen, R. P., Koelle, E. S., *J. Pharm. and Exp. Therap.*, 1946, **87**, 85.

4 Braun, H. A., Lusky, L. M., and Calvery, H. O., *J. Pharm. and Exp. Therap.*, 1946, **87**, 119.

5 Durlacher, S. H., Bunting, H., Harrison, H. E., Ordway, N. K., and Albrink, W. S., *J. Pharm. and Exp. Therap.*, 1946, **87**, 28.

TABLE I.
Experiment with Selenium (Sodium Selenite, SS).*
Ten animals in each series.

| Sex | Drug administered and method | Dosage (mg/kg) | Survival time (days) |
|---|---|---|---|
| ♀ | S.S.–(I.P.) | 5 | All survived |
| ♂ | ,, | 5 | ,, ,, |
| ♀ | ,, | 10 | Avg—10 |
| ♂ | ,, | 10 | Avg— 6 |
| | Experiment with BAL (British Anti-Lewisite) | | |
| ♀ | BAL–(I.M.) | 15 | All survived |
| ♂ | ,, | 15 | ,, ,, |

* Age of all rats used, 6 wk.

TABLE II.
Experiment with BAL and Selenium.*

| No. of animals and sex | Drug administered and method | Dosage (mg/kg) BAL | SS | Survival time days (avg) |
|---|---|---|---|---|
| 8 ♀ | BAL (I.M.) + SS (I.P.) | 15 | 5 | 10 |
| 8 ♂ | ,, | 15 | 5 | 6 |
| 8 ♀ | ,, | 7.5 | 5 | 6 |
| 8 ♂ | ,, | 7.5 | 5 | 5 |
| | (Using same animal after 20 days on BAL). | | | |
| 10 ♀ | BAL (I.M.) + SS (I.P.) | 15 | 5 | 3 |
| 10 ♂ | ,, | 15 | 5 | 3 |

* Age of all rats used, 6 wk.

a survival time of 5½ days ranging from 5 to 6 days (Table II). Microscopic examination of the liver showed no pathology but the kidneys exhibited a tubular necrosis.

2. Pre-treatment with BAL. Rats were pretreated 20 days with BAL, 15 mg/kg. On the 21st day and days after for as long as they lived, 5 mg/kg of SS was administered simultaneously with 15 mg/kg of BAL. The average survival time in this combined treatment was 3 days (Table II). Microscopic examination of the liver was normal but the kidney again exhibited moderate necrosis (Fig. 2).

*Discussion.* When SS was used alone a typical toxic picture was noted.[6] The livers of these animals were necrotic and showed moderate to severe fatty degeneration.

BAL alone did not produce any untoward results in our experience although Durlacher *et al.*[5] reported toxic reactions in mammals from the doses we used. The results of the combined doses of BAL and selenium led to a marked decrease in the survival time of the animals treated. This finding corresponds to the report of Braun, Lusky, and Calvery.[4] However, these investigators did not report any microscopic examination of the liver and kidney.

In our work, careful examination of the liver and kidney showed that, contrary to the usual findings in SS poisoning, there were no abnormalities in the liver tissue although the animals died rapidly. This might be regarded as due to the short survival time but it was noted that animals treated with SS alone for a period of 4 to 5 days *did* show the beginning of liver necrosis.

The results of our experiments would seem to indicate that the liver damage and death produced by SS may be entirely separate phenomena. As evidence in support of this contention BAL plus SS decreased the survival time of all animals as compared to those receiving SS alone. However, BAL protected SS animals from the liver damage usually produced by SS.

Seemingly, BAL does not antagonize the total toxicity of SS as it does other metals. However, it appears fair to propose that certain SH groups furnished by BAL prevent the liver damage caused by SS but are incapable

6 Smith, M. I., Strohlman, E. F., and Lillie, R. D., *J. Pharm. and Exp. Therap.*, 1937, **60**, 449.

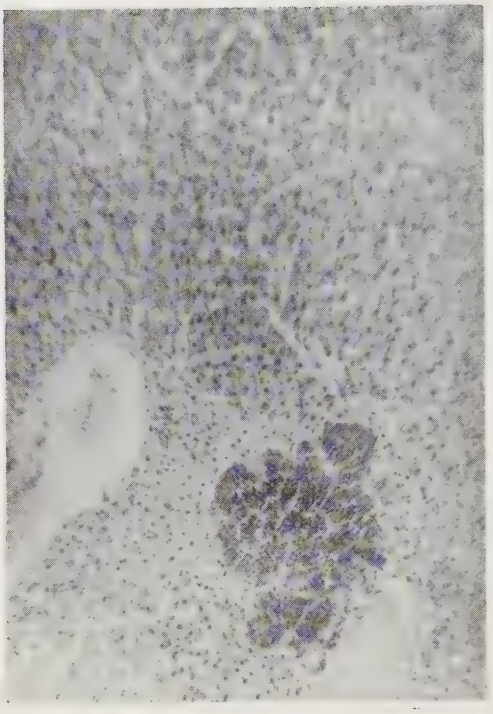

FIG. 1.
Rat liver: Typical focal necrosis and fatty degeneration following the administration of selenium in doses of 5 mg/kg for 10 days.

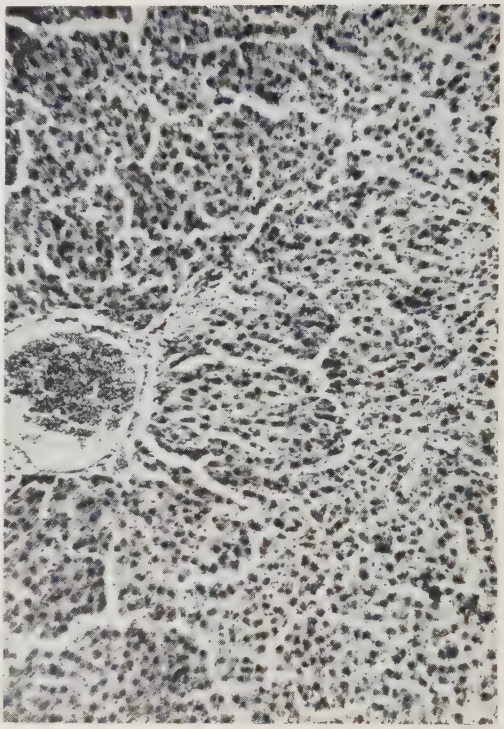

FIG. 2.
Rat liver: No abnormalities, following simultaneous administration of BAL 15 mg/kg and SS 5 mg/kg for 10 days.

of completely detoxifying the actions of SS.

It is now evident that BAL diminishes the toxicity of selenium for certain vital tissues and enhances the toxicity of the metal for the kidney. This corresponds to the findings of Gilman *et al.*,[3] who showed that cadmium becomes nephrotoxic with BAL. There are now evidently two such metals that have this property.

Our thanks are due Doctor R. L. Ferguson, Professor of Pathology, for reporting the microscopic findings in this paper.

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## A Limited Feeding Regime for Rats.* (17377)

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For the past 5 years, a rat feeding regime has been in use by this laboratory which has proved so helpful under varied circumstances as to warrant this brief presentation. In carrying out metabolic research, some baseline of activity is needed. The definition of the "post-absorptive condition" has met this need in the human and dog, essentially daytime animals. However, the nocturnal feeding habits of the laboratory rat have led to the

* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

establishment of a 24-hour fast as the standard procedure, in which food is removed from the cage in the morning and the animals used the next morning. Considerable data have been accumulated on this preparation, and it will continue to be a very useful acute one. However, one great disadvantage of such a procedure is that it should not be repeated within a week if the animals are to be maintained in good condition. This restriction makes it impossible to study in each animal the time-curve of response to an experimental procedure and when used chronically over a period of weeks may seriously debilitate a high proportion of the rats.

Since rats learn readily, it was considered feasible to attempt to train them to consume their food from 5 to 10 P.M., so that they would be post-absorptive 15 hours later, 1 P.M. on the following day. Oxygen consumption or other metabolism studies could then be made from 1 to 5 p.m. without in any way interfering with the 5 to 10 feeding period for that day. The findings fulfulled the expectations.

*Experimental.* For most work in this laboratory, 4 adult rats are kept in a metal cage 20″ x 16″ x 12″. The food, in checkers about 1″ x 1″ x ½″ (Purina Dog Chow, Purina Laboratory Chow, Rockland Rat Diet), is fed from metal containers with the front and bottom out of ¼″ mesh hardware cloth. The animals have no trouble biting off pieces of about ⅛″ to ⅜″ in diameter, which they immediately eat from their own forepaws. These food checkers have also been ground to pass a 16 mesh screen and this fed from two open-top, cup-type containers per cage with equally satisfactory results. After removing the food containers, one should search the sawdust, paper strips, or other litter used on the floor of the cage for any stray pieces of food which could be consumed by the rats after the 10 P.M. deadline.

Oxygen consumption measurements were made in a modified Benedict[1] 4-unit closed-circuit apparatus.

*Results and discussion.* Twelve male and 12 female rats were placed on the feeding routine outlined when 120 days of age, while an equal number of littermate controls were kept on *ad lib* feeding. Fig. 1 reveals that these young adult animals were able to maintain their body weights after a preliminary period of about 2 weeks for adjustment to the schedule. Body weight measurements are

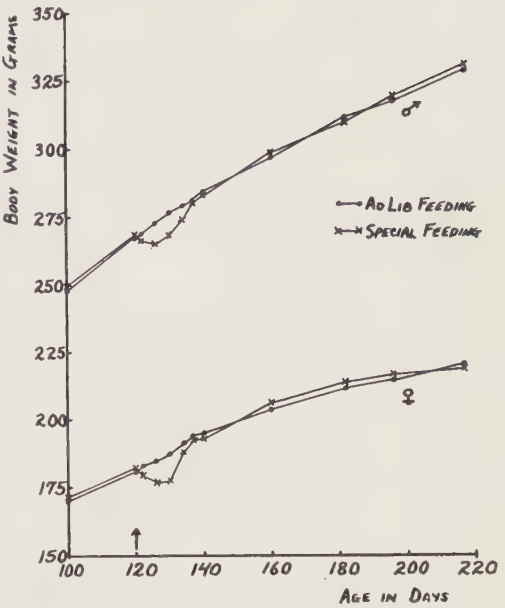


FIG. 1.
Body weight changes in rats placed on 5-hour feeding regime at 120 days of age.

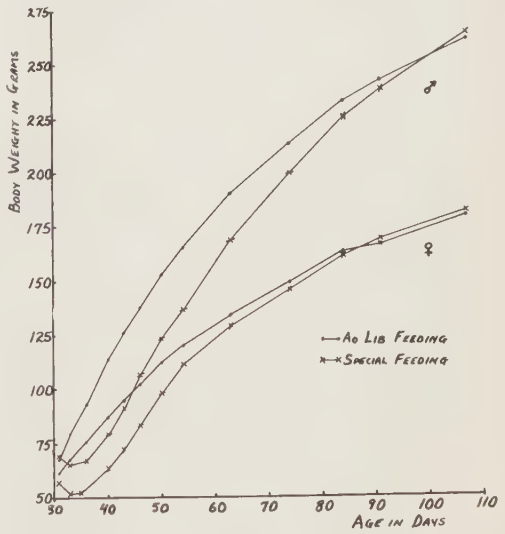


FIG. 2.
Growth curves of rats placed on 5-hour feeding regime upon weaning at 31 days of age.

[1] Benedict, F. G., *J. Nutrition*, 1930, **3**, 161.

TABLE I.
Metabolic Rates of Rats Fasted 24 Hours and of Rats on Special Feeding Regime.

| | cc $O_2$/100 g/hr | |
|---|---|---|
| | 24-hr fast | special regime |
| 28 ♂ | 100.9 | 101.3 |
| 20 ♀ | 102.8 | 102.7 |
| Avg of all 48 | 101.7 ± 4.66* | 101.9 ± 4.82* |

* Standard deviation.

probably more accurate when taken in the afternoon on this regime than on *ad lib* feeding, since occasional animals in the latter group eat during the daytime.

Even weanlings placed on the 5-hour feeding can eventually match the growth curves of littermates fed *ad lib*, as is shown in Fig. 2. In this experiment, groups of eight rats were weaned at 31 days of age from their mothers to ground rat food, available either *ad lib* or between 5 and 10 P.M. In these instances, the considerably longer time of nearly 2 months was required for complete readjustment, probably because of the increased requirements imposed by growth. Similar data are available for weanlings given their food in checker form.

Metabolic rates were compared in 48 adult rats, first after the conventional 24-hour fast, and then 3 to 6 weeks after being transferred to the limited feeding period regime. Table I shows that the two sets of oxygen consumption data were essentially identical. No apparatus was available for determining $CO_2$ production, so no R.Q.'s have been obtained for comparison. One would anticipate lower values after the extra 9 hours without food.

Although not shown because the results were not significantly different from those already discussed, 24 adult rats were trained to eat from noon to 5 P.M. so as to be ready for metabolism determinations at 8 A.M. on the next day. Even this remarkable alteration of habits, which essentially made daytime animals out of nocturnal ones, was successfully met.

Tepperman *et al.*[2] and Dickerson *et al.*[3] have shown that shortening the daily feeding time of rats to one hour resulted in such a distorted appetite in relation to caloric needs that the animals became obese. No such alteration in eating pattern has ever been observed in our animals. One may conclude from this that 5 hours' feeding time places no strain on the rat's ability to satisfy its appetite. No attempt was made to determine whether any of the alterations in carbohydrate metabolism noted by these authors could be shown to exist in our animals.

*Summary.* It has been found possible to train rats to eat their usual amounts of food in a 5-hour period, between 5 and 10 P.M. Fifteen hours later, at 1 P.M. on the following day, the animals are in a post-absorptive condition, prepared for a variety of metabolism studies.

The animals can be maintained indefinitely on this regime and the debilitating results of repeated periods of fasting for 24 hours can be avoided.

[2] Tepperman, J., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. Med.*, 1943, **15**, 855.

[3] Dickerson, V. C., Tepperman, J., and Long, C. N. H., *Yale J. Biol. Med.*, 1943, **15**, 875.

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# Effect of Chronic Administration of Mercurial Diuretics on Glomerular Filtration in the Dog.* (17378)

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The acute toxic effects of mercurial diuretics on the heart when administered intravenously are well known.[1-4] Recent reports on Thiomerin, the di-sodium salt of N($\gamma$-carboxymethylmercaptomercuri, $\beta$-methoxy) propyl camphoramic acid, have stressed the low cardiac[5,6] and local[7] toxicity when this mercurial diuretic is administered subcutaneously. Enthusiastic support has been given by several authors[8-11] for clinical use of this drug on cardiac patients.

In the following report, we have determined the effect of 3 mercurial diuretics, Thiomerin, meralluride (Mercuhydrin), and mersalyltheophylline (Merthyl) on glomerular filtration rate in the dog when these compounds are administered intravenously in comparable daily doses.

*Methods.* Control creatinine clearances were determined on each trained unanesthetized adult female dog one hour after hydration with 40 cc/kg of water by stomach tube. Creatinine determinations were performed by the method of Folin and Wu.[12] A series of 10 dogs were tested with Thiomerin, nine with Mercuhydrin, and 5 dogs were given Merthyl. The diuretics were administered intravenously in a dosage of 0.1 cc/kg/day (0.39 mg Hg/kg/day) for a period of 21 to 28 days. Creatinine clearances were determined at weekly intervals during the test period and in some instances were continued for one or two weeks after drug administration had been discontinued. In addition, mercury excretion determinations were made on three dogs of the Thiomerin series.

*Results.* Comparison was made of weekly changes in creatinine clearances of the dogs in each drug series. These changes were calculated in terms of percentage increase or reduction in the weekly creatinine clearance of each dog as compared to its control clearance level (Fig. 1, 2 and 3). It was observed that the creatinine clearances in many of the test animals were significantly reduced at the end of the third week of drug administration. However, there was noted a distinct trend toward return to the control clearance level during the recovery period in those animals which were followed for one or two weeks after the drug administration was discontinued.

Comparing the mean percentage changes from the control clearances of the dogs in each series indicates that in the third week Thiomerin produced a significantly greater reduction of creatinine clearance than the other mercurial diuretics tested (Fig. 4).

Evidence of toxicity from the mercurial diuretics developed early in some of the test animals. Three dogs in the Thiomerin series

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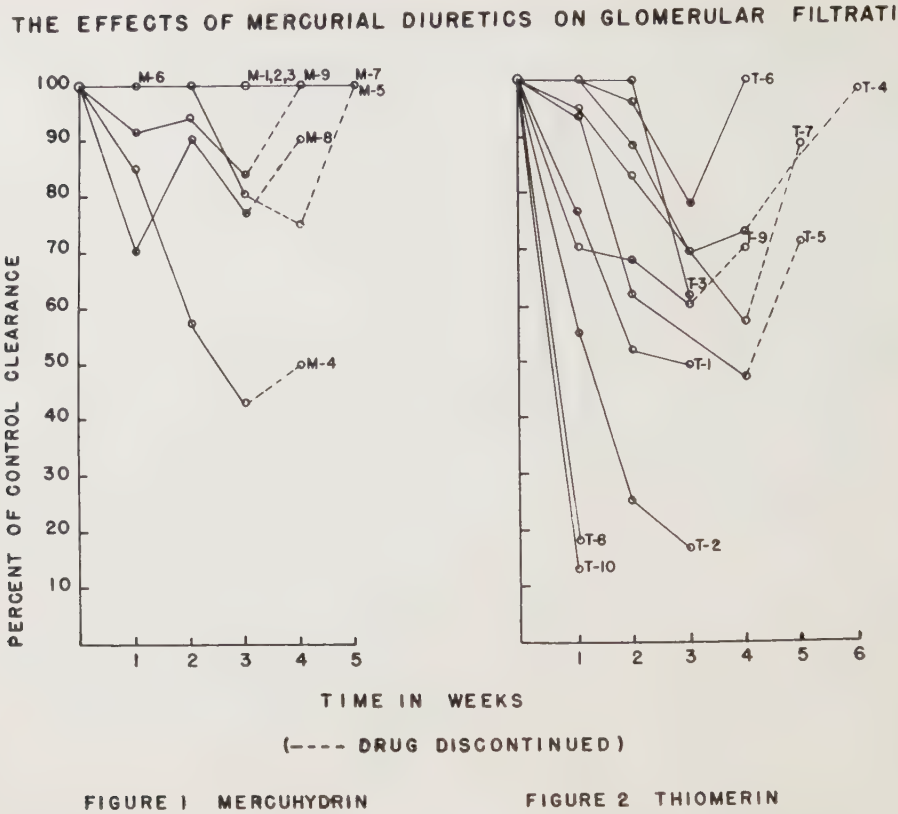


FIGURE 1 MERCUHYDRIN

FIGURE 2 THIOMERIN

(T-3, T-8, and T-10) developed a severe stomatitis in the first week of drug administration. Stomatitis did not occur in the dogs of the Mercuhydrin or Merthyl series. Moderate to severe diarrhea occurred in 4 Thiomerin dogs (T-4, T-5, T-8 and T-10) and none appeared in the Mercuhydrin and Merthyl dogs. Severe dermatitis with generalized loss of hair developed in two Thiomerin dogs (T-6 and T-7) and in one Mercuhydrin dog (M-8). One Thiomerin dog, not included in the series, died on the seventh day and two other Thiomerin dogs (T-8 and T-10) died on the 8th day with evidence of severe mercurial poisoning. A fourth Thiomerin dog (T-6) died with acute pulmonary edema four days after the drug was discontinued. One of the Mercuhydrin dogs (M-6) died on the seventh day of apparent acute cardiac toxicity immediately following intravenous injection of the drug. No deaths occurred in the Merthyl series.

*Discussion.* The dose employed in these experiments was chosen because it gives satisfactory diuresis in the dog for a period of 4-6 hours. When administered subcutaneously, a variable amount of mercury may be retained at the site of injection by local tissue combination, therefore the diuretics were given intravenously.

In general, evidence of mercurial poisoning was accompanied or heralded by an early reduction of the creatinine clearance. It would appear that the reduction in the glomerular filtration rate is a temporary manifestation if the drug is discontinued before irreparable renal damage is produced. In the dogs that had only mild signs of mercury poisoning, the creatinine clearance returned to normal one or two weeks after discontinuing the drug.

It appears that Thiomerin is more toxic than either Mercuhydrin or Merthyl when tested in this manner. Our opinion is based on the observation of evidence of mercury poisoning such as stomatitis and diarrhea which occurred more frequently in the Thiomerin dogs than

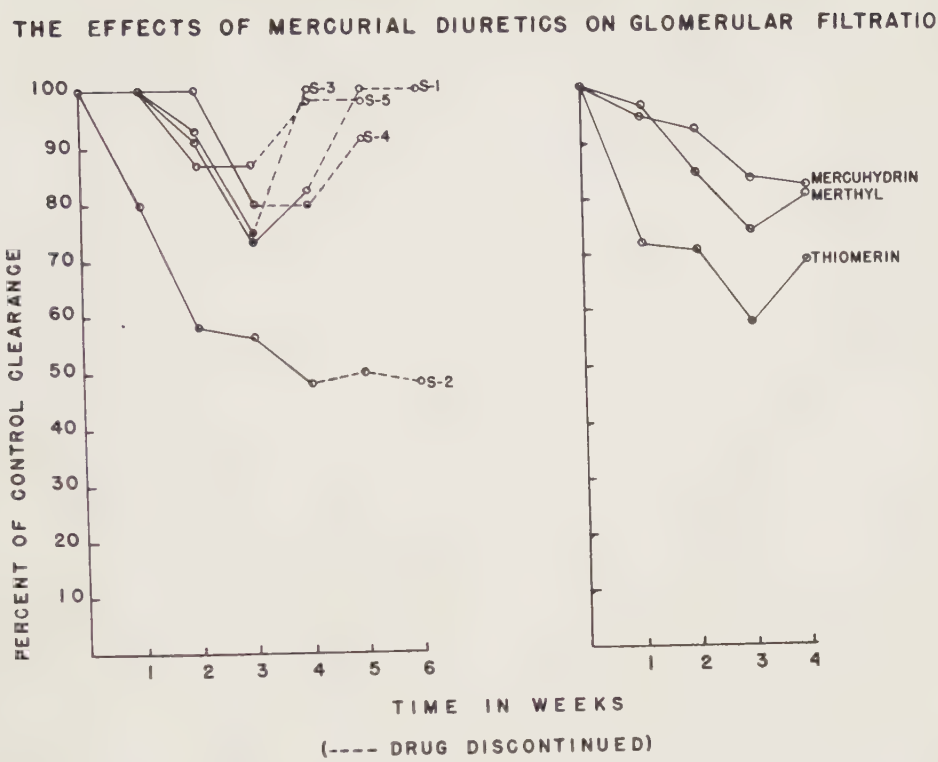


FIGURE 3 MERTHYL

FIGURE 4 MEAN CLEARANCE REDUCTIONS

in the animals given the other two drugs. We also noted that by the third week of drug administration, the creatinine clearances of the Thiomerin dogs were reduced to a greater extent than those of the dogs in the Mercuhydrin and Merthyl series. Special care was taken to use solutions of Thiomerin which were kept refrigerated and not over 4 days old.

Data obtained from mercury excretion determinations of 3 Thiomerin dogs suggested that the excretion of the drug is not as rapid or complete as with other mercurial diuretics. In the few studies conducted, the dogs that showed symptoms of mercury poisoning also showed a delayed excretion of mercury.

*Summary.* Mercurial diuretics administered intravenously in chronic dosage to dogs reduced the glomerular filtration rate as measured by the creatinine clearance. This reduction in glomerular filtration rate was found to be temporary in most dogs that did not have severe symptoms of mercury poisoning.

Thiomerin, when administered intravenously in comparable chronic dosage to the dog, was found to be of higher toxicity than either Mercuhydrin or Merthyl, as indicated by reduced creatinine clearances, and other signs of mercury poisoning.

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# Influence of Cells and Plasma on *In vitro* Survival of Malaria Sporozoites.* (17379)

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It has been shown that the sporozoites of *Plasmodium cathemerium 3H2,* infective for the canary but not the hen, retain their infectivity during several hours in drawn canary but not drawn hen blood.[1] The present communication reports investigation of the effect of the cell and plasma portions of these bloods upon the retention of infectivity.

*Mixtures of canary and hen whole bloods.* Infected mosquitoes were ground in Locke's solution and added to the following freshly prepared mixtures: (a) one-tenth canary and nine-tenths hen blood; (b) five-tenths canary and five-tenths hen blood; (c) nine-tenths canary and one-tenth hen blood. After incubation of the blood-sporozoite mixtures with gentle agitation at 41.5°C for 1½ hours, portions representing one mosquito were injected intramuscularly into canaries, the peripheral blood of these birds being subsequently examined from the 5th to 16th days, inclusive. Birds failing to become positive were proved negative in all instances by subsequent infection from mosquito bites. Trials were made at such intervals that in most instances at least one insectory cycle had been completed between them.

In the trials employing mixture (a), 27% of the birds became infected (see Table I); mixture (b) infected 72% and mixture (c) 87%.

*Canary and hen plasmas alone.* Sporozoites were separately incubated in canary and hen plasmas. In two trials (See Table I), they failed to retain infectivity in either plasma.

*Canary plasma alone and with canary cells.* Four trials were made of sporozoites in canary plasma alone and in another portion of the same plasma to which the normal amount of canary cells was returned after 3 washings in Locke's solution. None of the sporozoites retained their infectivity in the plasma alone (see Table I), but they did so in 100% of instances when the cells were present.

*Canary plasma with canary cells, hen cells, and both.* Seven trials were made of sporozoites in canary plasma to which the washed canary cells were returned, canary plasma to which an equivalent amount of washed hen cells were added, and canary plasma in which one-half the added washed cells were canary and the other half hen. When canary cells alone had been added, sporozoite infectivity was retained in 100% of instances (see Table I); when hen cells alone had been added, infectivity was lost completely; when the canary-hen cell mixture had been added, infectivity was retained to the extent of 74%.

*Hen plasma with canary cells, hen cells, and both.* These 5 trials constituted a test just the reverse of the above in that the fluid medium was hen instead of canary plasma. When hen cells were added there was no retention of sporozoite infectivity (Table I); when canary cells were added infectivity was retained 100%; when a mixture in equal parts of the two types of cells was added infectivity was 85% retained.

*Canary plasma with canary white and red cells, separately.* In 5 trials, after the initial separation of canary plasma and cells by centrifugation, the cells were washed 3 times in Locke's solution and then loaded through a long needle into the bulb and stem of a 2 cc Ostwald-Folin pipette modified by cutting off the delivery tip below the bulb, fusing the lower opening, and cutting down the suction stem to a size suited to the centrifuge. Spinning of this improvised centrifuge tube loaded with washed total blood cells delivered the white cells as "buffy layer" into the suction stem, whence they were removed with a thinly drawn capillary tube. One small drop of this buffy layer was blown into a 0.6 cc mixture,

* Research supported by National Institute of Health Research Grant 67 (C2).

1 Beckman, H., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 172.

TABLE I.
Survival of Malaria Sporozoites in Various Whole Blood, Plasma and Cell Mixtures.

| Trial No. | Mixtures of canary and hen whole bloods (C = canary, H = hen). 1/10 C-9/10 H (birds infected) | 5/10 C-5/10 H (birds infected) | 9/10 C-1/10 H (birds infected) |
|---|---|---|---|
| 1 | 1/4 | 2/3 | 4/4 |
| 2 | 3/5 | 5/5 | 5/5 |
| 3 | 2/5 | 4/4 | 5/5 |
| 4 | 1/5 | 3/5 | 3/5 |
| 5 | 0/8 | 7/8 | 8/8 |
| 6 | 1/8 | 1/8 | 5/7 |
| 7 | 4/8 | 7/7 | 6/7 |
| Totals | 12/43 (27%) | 29/40 (72%) | 36/41 (87%) |

| Trial No. | Canary and hen plasmas alone. C plasma (birds infected) | H plasma (birds infected) |
|---|---|---|
| 1 | 0/12 | 0/12 |
| 2 | 0/12 | 0/12 |
| Totals | 0/24 | 0/24 |

| Trial No. | Canary plasma alone and with Canary cells. Alone (birds infected) | With C cells (birds infected) |
|---|---|---|
| 1 | 0/6 | 6/6 |
| 2 | 0/6 | 6/6 |
| 3 | 0/6 | 6/6 |
| 4 | 0/6 | 6/6 |
| Totals | 0/24 (0%) | 24/24 (100%) |

| Trial No. | Canary plasma with Canary cells, Hen cells, and both. With C cells (birds infected) | With H cells (birds infected) | With C and H cells (birds infected) |
|---|---|---|---|
| 1 | 5/5 | 0/5 | 4/5 |
| 2 | 5/5 | 0/5 | 2/5 |
| 3 | 5/5 | 0/5 | 1/5 |
| 4 | 4/4 | 0/4 | 4/4 |
| 5 | 8/8 | 0/7 | 8/8 |
| 6 | 7/7 | 0/7 | 6/8 |
| 7 | 8/8 | 0/8 | 7/8 |
| Totals | 42/42 (100%) | 0/41 (0%) | 32/43 (74%) |

| Trial No. | Hen plasma with Canary cells, Hen cells, and both. With C cells (birds infected) | With H cells (birds infected) | With C and H cells (birds infected) |
|---|---|---|---|
| 1 | 4/4 | 0/4 | 4/4 |
| 2 | 4/4 | 0/4 | 4/4 |
| 3 | 4/4 | 0/4 | 1/4 |
| 4 | 4/4 | 0/4 | 4/4 |
| 5 | 4/4 | 0/4 | 4/4 |
| Totals | 20/20 (100%) | 0/20 (0%) | 17/20 (85%) |

| Trial No. | Canary plasma with Canary white and red cells, separately. White cells (birds infected) | Red cells (birds infected) | Plasma alone (birds infected) |
|---|---|---|---|
| 1 | 5/5 | 3/5 | 0/5 |
| 2 | 5/5 | 3/5 | 0/5 |
| 3 | 5/5 | 3/5 | 1/5 |
| 4 | 8/8 | 3/8 | 0/8 |
| 5 | 5/7 | 1/8 | 0/8 |
| Totals | 28/30 (93%) | 13/31 (41%) | 1/31 (3%) |

half of which was canary plasma and half Locke's solution containing the sporozoites. One small drop of red cells obtained from deep down in the bulb of the pipette was then

blown into another portion of the same plasma-Locke-sporozoite mixture. Incubation and subsequent injections as usual.

Survival of sporozoite infectivity occurred in 93% of instances when white cells alone had been returned to the plasma (see Table I); in 41% when red cells alone had been added; in 3% when presumably plasma alone was present.

*Discussion.* In the recent interesting paper of McGhee,[2] who succeeded in infecting Swiss mouse erythrocytes injected intravenously into chick embryos 2 days after infecting the embryos intravenously with *P. lophurae* trophozoites, it is suggested that perhaps the malaria resistant principle in certain species lies in the serum constituents rather than in the cells. The studies herein reported indicate that the reverse is the case with regard to sporozoite survival, at least in drawn blood. The sporozoites in these experiments did not survive in the plasma of either the resistant or the susceptible species, but they survived equally well in the plasma of either species provided cells of the susceptible species were present. They did not survive, however, in the plasma of the susceptible species if cells of the resistant species alone were added, though they survived extremely well in the plasma of either species if, in addition to cells of the resistant species, cells of the susceptible species were present. Apparently, therefore, insofar as one can draw conclusions from a study of two species of the same phylum, the survival of sporozoites in drawn blood depends upon the presence therein of cells of the susceptible species. This would appear to conform to the *in vivo* observation of Huff and Coulston,[3] who were unable to prevent sporozoite infection of *P. gallinaceum*-susceptible chickens by inoculation of them with the whole blood, serum or plasma of non-susceptible ducks, but who did succeed in conveying to ducks some of the susceptibility of the chicken by inoculating them with chicken blood.

Whether it is the white or red blood cells of the susceptible species which make survival possible was not conclusively determined in these studies, though the evidence (Table I) favors the greater importance of the white cells. If a technic can be evolved which will deliver pure samples of white and red cells, it should be possible to settle the point. Contamination of the white cells with a small number of red cells would not probably be important in view of the evidence, but it appears that the presence of only a few white cells in the red cell sample could considerably facilitate survival because it required only one small drop of the buffy layer in 0.6 cc of diluent to enable practically complete survival to take place.

No evidence of the nature of the action of the canary cells in facilitating sporozoite survival *in vitro* was obtained. Prolonged study of stained smears made at various times during the incubation of the mixtures did not reveal sporozoites or anything resembling pre-erythrocytic forms in any of the cells. Immunologic studies were not performed.

*Summary.* In a study of the role of cells and plasma in retention of infectivity of sporozoites of *P. cathemerium 3H2* in canary blood and the failure of such retention in hen blood, it was found that (a) survival occurs in hen whole blood provided a small amount of canary whole blood is present; (b) it does not occur in either canary or hen plasma alone; (c) it occurs in either canary or hen plasma if canary cells are present whether or not hen cells are simultaneously present but does not occur in either plasma if only hen cells are present; (d) it occurs in canary plasma to a greater extent if canary white cells (somewhat contaminated with canary red cells) are present than if canary red cells (somewhat contaminated with white cells) are present. The possible significance of the findings is discussed.

Technical assistance of Darrell B. Paul is gratefully acknowledged.

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[2] McGhee, R. B., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 92.

[3] Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1946, **78**, 99.

# Toxicity of Sodium Tetrathionate. (17380)

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(Introduced by H. B. Haag.)

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Sodium tetrathionate monohydrate ($Na_2S_4O_6 \cdot H_2O$) was first proposed by Theis and Freeland[1] for use in the treatment of thrombo-angiitis obliterans. Their object was to provide a greater and more prolonged effect than that obtained by sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$). These substances comprise an oxidation-reduction system but thiosulfate will not oxidize SH groups while tetrathionate does have this action. It has been suggested by Philips *et al.*,[2] Goffart and Fischer[3] that the kidneys may be damaged by this fundamental reaction.

The Council on Pharmacy and Chemistry of the American Medical Association[4] quoted the work of Chen, Rose, and Clowes,[5] also that of Gilman and his associates,[6] on the toxicity of this substance. It was stated that the intravenous $LD_{50}$ in the dog was 250 mg per kilo and in the rabbit 75 mg per kilo. The animals died from renal failure due to severe necrotizing lesions in the proximal convoluted tubules. A later report of the Council consisted of communications from Theis and DeTakats,[7] who emphatically stated that the clinical dose of sodium tetrathionate was only a small fraction of the lethal dose in animals. They advocated a review of the toxicity of this drug from a clinical standpoint.

In an effort to determine the toxicity of sodium tetrathionate (Tetrathione, Searle) clinically, we decided on the following experiment.

*Method of study.* A group of 30 patients, admitted to the Medical Service of the Medical College of Virginia, Hospital Division, some with peripheral vascular disease, and others with various other maladies, were subjected to routine blood non-protein nitrogen, Mosenthal concentration and phenolsulphonphthalein tests. These patients were then given 0.6 g (10 cc) of tetrathione, intravenously twice daily, for a period of 7 days. This dosage was equivalent to that of the usual course of treatment of peripheral vascular disease, lasting 7 weeks. At the end of the 7 day period, the non-protein nitrogen, Mosenthal concentration, and phenolsulphonphthalein studies were repeated. The results are given in Table I.

There were no untoward reactions to the drug. None of the patients manifested nausea, vomiting, abdominal pain, or weakness, which have been reported following too rapid injection or during the course of clinical treatment. The changes in the laboratory findings were mostly of a minimal character. In 15 cases the non-protein nitrogen showed a slight increase, in 8 cases there was a slight decrease, and in 7 cases there were no changes. The Mosenthal concentration test showed an increase in specific gravity in 10 cases, a decrease in 7 cases, and in 13 cases there were no changes. The phenolsulphonphthalein test showed an increased concentration of the dye in 6 cases, in 15 cases there was less concentration, and in 9 cases there were no changes. The maximum increase in the non-protein nitrogen was from 20 mg% to 35 mg% in case 18, a patient with thrombo-angiitis obliterans. We consider 35 mg % within the normal range. The Mosenthal concentration test in case 6 showed a variation from a maximum specific gravity of 1.032 prior to tetrathione to 1.018 follow-

1 Theis, F. F., and Freeland, M. R., *Arch. Surg.*, 1940, **40**, 190.

2 Philips, F. S., Gilman, A., Koelle, E. S., and Allen, R. P., *J. Biol. Chem.*, 1947, **167**, 209.

3 Goffart, M., and Fischer, P., *Archives Internationales de Physiologie*, 1948, **55**, 258.

4 Council on Pharmacy and Chemistry, *J.A.M.A.*, 1947, **133**, 693.

5 Chen, K. K., Rose, C. L., and Clowes, G. H. A., *Am. J. Med. Sci.*, 1937, **188**, 767.

6 Gilman, A., Philips, F. S., Koelle, E. S., Allen, R. P., and St. John, E., *Am. J. Physiol.*, 1946, **147**, 115.

7 Supplemental Report on Sodium Tetrathionate, *J.A.M.A.*, 1947, **134**, 1092.

TABLE I.
Sodium Tetrathionate Experiments.

| Case No. | P.S.P. control, % | P.S.P. after, % | N.P.N. control, mg % | N.P.N. after, mg % | Mosenthal (highest) Control | Mosenthal (highest) After |
|---|---|---|---|---|---|---|
| 1 | 80 | 63 | 24 | 32 | 1.022 | 1.022 |
| 2 | 85 | 75 | 23 | 35 | 1.024 | 1.020 |
| 3 | 60 | 55 | 36 | 27 | 1.022 | 1.020 |
| 4 | 55 | 55 | 28 | 38 | 1.022 | 1.025 |
| 5 | 70 | 60 | 33 | 32 | 1.022 | 1.020 |
| 6 | 60 | 70 | 30 | 33 | 1.032 | 1.018 |
| 7 | 58 | 50 | 26 | 30 | 1.020 | 1.018 |
| 8 | 55 | 55 | 25 | 30 | 1.018 | 1.016 |
| 9 | 60 | 55 | 35 | 35 | 1.018 | 1.018 |
| 10 | 45 | 50 | 30 | 27 | 1.020 | 1.020 |
| 11 | 60 | 60 | 44 | 40 | 1.015 | 1.016 |
| 12 | 83 | 65 | 23 | 27 | 1.013 | 1.016 |
| 13 | 60 | 53 | 29 | 27 | 1.017 | 1.018 |
| 14 | 90 | 62 | 23 | 27 | 1.020 | 1.018 |
| 15 | 55 | 55 | 32 | 27 | 1.022 | 1.022 |
| 16 | 55 | 55 | 41 | 32 | 1.017 | 1.016 |
| 17 | 75 | 60 | 33 | 29 | 1.026 | 1.026 |
| 18 | 51 | 34 | 20 | 35 | 1.016 | 1.023 |
| 19 | 70 | 60 | 25 | 27 | 1.012 | 1.012 |
| 20 | 53 | 50 | 24 | 24 | 1.012 | 1.017 |
| 21 | 47 | 45 | 30 | 33 | 1.012 | 1.012 |
| 22 | 50 | 45 | 31 | 36 | 1.016 | 1.015 |
| 23 | 40 | 45 | 36 | 36 | 1.012 | 1.021 |
| 24 | 50 | 58 | 33 | 30 | 1.021 | 1.018 |
| 25 | 105 | 55 | 27 | 26 | 1.018 | 1.016 |
| 26 | 75 | 70 | 26 | 37 | 1.012 | 1.019 |
| 27 | 60 | 60 | 32 | 30 | 1.022 | 1.022 |
| 28 | 70 | 60 | 47 | 50 | 1.020 | 1.022 |
| 29 | Incomplete | | | | | |
| 30 | 40 | 45 | 36 | 37 | 1.014 | 1.012 |
| Avg | 63 ± 2.7 | 57 ± 1.7 | 30 ± .11 | 32 ± 10 | 1.018 ± .009 | 1.019 ± .0006 |

ing the drug. In all other cases the variation in Mosenthal concentration was minimal in either direction. The phenolsulphonphthalein test showed the most marked variations. It was decreased by 18% in case 12 and decreased by 27.5% in case 14. The most unusual change was in case 25, in which there was a decrease of 50%. There was no correlation in any separate case between the changes in the various tests.

A statistical analysis[8] of the data is outlined in Table I. It will be noted that the phenolsulphonphthalein control excretion 63.1% ± 2.75. After sodium tetrathionate the excretion was 56.8% ± 1.69. The "t" value for the difference of these means is 1.955 which, with a normal deviate of 1.96, gives a probability of 0.05. The values for the non-protein nitrogen before and after sodium tetrathionate were 30.3 mg% ± .115, and 32.1 mg% ± 0.97. This indicates no significant difference. The Mosenthal values consider only the highest concentration obtained. They were 1.018 ± 0.009 and 1.019 ± 0.0006. This indicates very little difference. The statistical analysis demonstrates that for the dosage employed sodium tetrathionate does not materially affect kidney function as regards the non-protein nitrogen and Mosenthal tests. The phenolsulphonphthalein excretion might be interpreted as definitely reflecting some changes in kidney function.

*Summary.* Thirty patients were chosen at random from the routine admissions to the Medical Service of the Medical College of Virginia Hospital Division and given 0.6 g sodium tetrathionate twice daily for 7 days, intravenously. The effect on kidney function was studied by following changes in blood non-protein nitrogen, Mosenthal concentration and phenolsulphonphthalein excretion values. The blood non-protein nitrogen and the Mos-

[8] Snedecor, G. W., Statistical Methods, fourth edition, Iowa State College Press, 1946.

enthal concentration values indicated little disturbance in kidney function. However, these tests would not be expected to show much change unless there was a rather extensive impairment in renal function. The phenolsulphonphthalein excretion test was our most sensitive test of tubular function and in several instances showed evidence of diminished tubular capacity following sodium tetrathionate administration. On the basis of these clinical studies it would seem that sodium tetrathionate probably should not be used if there is any pre-existing renal disease.

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## Glycogen in Basophilic Leucocytes in Human Blood Smears. (17381)

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In a recent study of the occurrence of the periodic acid-Schiff reaction in various normal cells of the blood and connective tissue by Wislocki, Rheingold and Dempsey,[1] small clear red dots were observed in the pale pink cytoplasm of the basophilic leucocytes. These investigators were not able to identify the basophiles in control blood smears digested by saliva. They therefore concluded that the red stained material in the undigested smears may have been glycogen but that the "apparent finding needs further verification." Similarly, Gibb, and Stowell[2] decided that "Because myeloid cells which were free from glycogen were not observed in normal peripheral blood and bone marrow films, basophils, although not specifically identified, may also contain glycogen."

To make certain that basophils could be identified in smears digested by saliva before treatment with periodic acid and leucofuchsin, dried smears of human blood were stained in Wright's blood stain in absolute methyl alcohol for 3 minutes. This step was not followed by the usual one of the diluted stain. For the most part, the smears were very quickly rinsed in 95% ethyl alcohol although at the beginning of the study a few were dipped in running water. Basophils were located and each cell was ringed by means of an object marker. A map was made giving its position in the circle. The smears were then fixed in absolute ethyl alcohol for 5 minutes, rinsed in 95% and 70% alcohol and stained by the periodic-leucofuchsin technic of McManus[3] with ½% periodic acid, or, as modified by Wislocki, Rheingold and Dempsey,[1] with 1% periodic acid. The smears to be digested were covered with saliva for 45 to 60 minutes after fixation in absolute alcohol and rinsed in running water. After the smears had been examined with no nuclear stain, they were stained in Mayer's hematoxylin for 10 minutes.

In the undigested smears, the glycogen in the neutrophiles was stained a deep to pale red-pink, either evenly throughout the cytoplasm, or more deeply either around the periphery of the cell or off at one side. The 29 basophilic leucocytes examined were, in contrast, much paler, a difference seen clearly when the two kinds of cells were found next to each other. There was a marked variation in the amount of glycogen in the basophiles even on the same slide. In some cells rather large clear red granules were seen in groups with finer grains distributed more regularly among the unstained basophilic granules. In others only these dust-like particles of glycogen were visible. A third variety had a hazy pink background which outlined the colorless basophilic granules A few had no color at all.

1 Wislocki, G. B., Rheingold, J. J., and Dempsey, E. W., *Blood*, 1949, **4**, 562.

2 Gibb, R. P., and Stowell, R. E., *Blood*, 1949, **4**, 569.

3 McManus, J. F. A., *Stain Tech.*, 1948, **23**, 99.

In the digested smears the cytoplasm of the 12 basophiles studied was almost transparent and without color. It is therefore concluded that the material in the basophilic leucocytes which stained red in leucofuchsin after periodic acid is glycogen. Furthermore, since glycogen can be demonstrated in the human basophilic leucocytes, they do "differ from mast cells which contain periodic acid-Schiff positive material which is insoluble in saliva." (Wislocki, Rheingold and Dempsey[1]).

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## A Quantitative Method for Measuring Staphylococcal Anticoagulase.* (17382)

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The study of infections produced by *Staphylococcus aureus* in man or in experimental animals has been hampered by the lack of a suitable serological test. Since staphylococcal coagulase will stimulate the production of an inhibitor substance, termed anticoagulase,[1,2] it has been possible to devise a serological test that could be used in the study of infections caused by this organism.

The mechanism whereby staphylococcal coagulase clots plasma has been partially clarified by the recent work of Smith and Hale,[3] Kaplan and Spink,[4] and Tager.[5] Most strains of *S. aureus* produce an extracellular substance, coagulase, which, added to plasma, results in the formation of a clot. Coagulase does not clot fibrinogen directly, for it has been found that a second factor, termed activator[3] or reacting factor,[5] is also required. This substance is found in the blood of man and of certain animal species. Although it is not entirely clear at the moment whether coagulase, activator, or a product of these two substances is responsible for the clotting of fibrinogen, antibodies against coagulase develop following immunization with cell-free coagulase. By employing measured amounts of coagulase, fibrinogen and activator, it has been possible to develop a quantitative method for the measurement of coagulase inhibitor, anticoagulase.

*Materials and methods. Buffered Saline.* A solution of 0.01 M phosphate in 0.85% sodium chloride at a pH of 7.4 was prepared for dilution of all sera and for preparation of fibrinogen, activator and heparin solutions.

*Activator.* The source of activator was 300 ml of human serum from one donor. This standard serum was stored in glass containers containing 50-75 ml at 4°C.

Two strengths of activator were employed. For standardization of coagulase, a final dilution of 1:3500 of the serum in a solution of fibrinogen was employed. In the coagulase inhibition test a dilution of standard serum of 1:1000 was used.

*Heparin.* A solution of heparin containing 100 mg per 10 ml of buffered saline solution was prepared and stored at 4°C until used. A 1:100 dilution of this was added to the fibrinogen-activator mixture.

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1 Rammelkamp, C. H., *Am. J. Med.*, 1948, **4**, 782.

2 Tager, M., and Hales, H. B., *J. Immunol.*, 1948, **60**, 475.

3 Smith, W., and Hale, J. H., *Brit. J. Exp. Path.*, 1944, **25**, 101.

4 Kaplan, M. H., and Spink, W. W., *Blood*, 1948, **3**, 573.

5 Tager, M., *Yale J. Biol. and Med.*, 1948, **20**, 269.

*Coagulase.* Cell-free coagulase was prepared as follows: From 5-10 ml of an overnight culture of a coagulase-positive strain of *S. aureus* in tryptose broth were placed in a flask containing 500 ml of tryptose broth to which 10 ml of bovine albumin† had been added. This broth culture was incubated for 72 hours at 37°C, following which the cells were sedimented in a refrigerated centrifuge for 2 hours at 2,000 RPM. The supernate was removed and stored at 4°C overnight. The supernate was then passed through a Seitz filter, the first 50 ml being discarded. The filtrate was tested for sterility and stored in the frozen state in ampules. At the time of use it was thawed rapidly and the necessary dilutions made with sterile tryptose broth.

*Fibrinogen.* For standardization of coagulase a solution of 100 mg of Fraction I (bovine fibrinogen‡) in 63 ml of buffered saline solution was employed. After incubation at 37°C for 30 minutes with occasional agitation, 1 ml of the 1:100 dilution of heparin was added.

To 50 ml of this solution was added 0.1 ml of the standard activator. Using volumetric pipettes, this was further diluted with the fibrinogen solution so that the final concentration of activator was 1:3500.

For the coagulase-inhibition test, 100 mg of fibrinogen was dissolved in 31 ml of buffer and following incubation at 37°C for 30 minutes, 1 ml of 1:100 heparin was added. Activator was added to this solution so that there was a final concentration of 1:1000.

*Serum.* Blood was collected and allowed to clot at room temperature. After separation, the serum was stored either at –20°C or at 4°C. The day before testing, 0.25 ml of sera were placed in small tubes and inactivated in a water bath at 60°C for 20 minutes. The sera were then stored at 4°C for 60 minutes and again inactivated at 60°C for 20 minutes. To each serum was added 6 ml of buffered saline solution and the solution was stored at 4°C overnight.

*Standardization Procedure.* A preliminary standardization of the coagulase preparation is performed by making serial two-fold dilutions in 1 ml volumes, using tryptose broth as a diluent. To each tube is added 1 ml of the fibrinogen solution containing 1:3500 activator. The rack containing the tubes is shaken, and placed in the 37°C water bath for 3 hours. The preliminary titer is then read as the highest dilution of coagulase which results in the formation of a visible clot.

The final titration employs the proper dilution of coagulase in tryptose broth as indicated by the preliminary titration. The coagulase is added to a duplicate series of 6 tubes in decrements of 0.1 ml. Tryptose broth is added to bring the volume up to 1 ml.

One ml of the fibrinogen standard is added, and incubation carried out as above. A unit of coagulase is then defined as the smallest amount which results in the slightest visible shred of fibrin after 3 hours' incubation. Reading of the test is facilitated by the use of a hand lens.

*Coagulase-inhibition Test.* Previous studies[6,7] have indicated that the union of coagulase and inhibitor is practically complete after 90 minutes' incubation at 37°C. The procedure employed for the titration of anticoagulase includes a preliminary and a fine titration as follows: to a series of 10 x 125 mm tubes containing 1 ml of various dilutions of serum previously inactivated as described above, is added 0.5 ml of coagulase containing 1 unit. After shaking, the mixtures are incubated at 37°C for 90 minutes and 0.5 ml of fibrinogen solution containing 1:1000 activator and heparin is added with an automatic pipette. The tubes are placed again in a water bath at 37°C for 3 hours. The titer of anticoagulase is read as the greatest dilution (initial) of serum which completely inhibits the formation of any visible shred.

In the preliminary titration the initial dilutions of serum employed are 1:25, 1:125, and 1:625. The final titer is then determined using 4 or 5 tubes of the proper dilutions as indicated in Table I.

*Accuracy.* To establish the accuracy of the

† Armour and Co.

‡ The authors wish to thank Armour and Co., Chicago, Ill., for supplying this product.

6 Lominski, I., and Roberts, A. B. S., *J. Path. and Bact.*, 1946, **58**, 187.

7 Rammelkamp, C. H., unpublished observations.

TABLE I.
Method for Preparing Preliminary and Fine Titrations of Sera for Anticoagulase Titrations.

| Preliminary dilution of serum | Serum, ml | Buffered saline, ml | Initial dilution |
|---|---|---|---|
| 1/25 | 1.0 | 0 | 25 |
| 0.25 ml of serum | 0.7 | 0.3 | 36 |
| + | 0.45 | 0.55 | 56 |
| 6.0 ml of buffered saline | 0.3 | 0.7 | 83 |
| 1/125 | 1.0 | 0 | 125 |
| 1.0 ml of 1/25 serum | 0.7 | 0.3 | 179 |
| + | 0.45 | 0.55 | 278 |
| 4.0 ml of buffered saline | 0.3 | 0.7 | 417 |
| 1/625 | 1.0 | 0 | 625 |
| 0.5 ml of 1/25 serum | 0.7 | 0.3 | 900 |
| + | 0.45 | 0.55 | 1400 |
| 12.0 ml of buffered saline | 0.3 | 0.7 | 2100 |

test 4 control sera are included in all titrations of anticoagulase. The results of repeated tests on 2 sets of control sera are shown in Table II. Titers of sera run on dffierent days varied only 2 dilution increments. It would appear that acute and convalescent sera exhibiting a difference in titer of 2 or more dilution increments, as determined on a single titration, represent a true difference in inhibitory substance. Occasionally it may be desirable to determine the amount of antibody in dilutions less than 1:25; in such cases the reproducibility of the titers is somewhat less accurate.

*Discussion.* The serological test described in the present report has been used for the past 2 years and numerous sera have been examined. The technic is simple; 100 or more sera may be titrated in one day. The test has the advantage that the various reacting substances are carefully controlled. A fixed amount of coagulase is allowed to react with dilutions of the serum to be tested, following which known amounts of fibrinogen and activator are added. The end-points are reproducible and easily read.

Living cultures of *S. aureus* have usually been employed in attempts to demonstrate an antibody to coagulase. Such a procedure precludes a quantitative measurement of the inhibitor since variable amounts of coagulase are produced during incubation of the plasma-coagulase mixtures. Cell-free coagulase may be obtained by heating or filtering cultures of *S. aureus*[8] or by the addition of bacteriostatic agents.[9] Variable results have been obtained as regards the resistance of coagulase to heat and filtration.[8] Acid cultures yield no coagulase upon heating, and alkaline cultures yield little or no coagulase on filtration. Lominski and Roberts[6] employed broth containing 10% plasma and found that coagulase would then pass through a filter. Originally we employed plasma in the culture medium, but later it was found that filtrates from such cultures clotted purified fibrinogen directly, indicating that a significant and variable amount of activator from the plasma remained in the culture filtrate. Since the measurement of coagulase is dependent upon the interaction of activator and coagulase, plasma cannot be used in the culture medium. For this reason bovine albumin was employed, and it was determined that coagulase became filterable in the presence of albumin.

Activator was obtained from one source: human serum from one donor known to be free of a coagulase inhibitor. Because this serum was capable of clotting plasma or fibrinogen solutions directly, heparin was added to the system. Experience has shown that most animal sera and an occasional human serum will clot fibrinogen directly. In the titration of antibody, sera to be tested are inactivated in order to remove the activator or reacting substance. It is important that inactivated

[8] Lominski, I., and Milne, J. A., *J. Path. and Bact.*, 1947, **59**, 516.

[9] Tager, M., and Hales, H. B., *Yale J. Biol. and Med.*, 1947, **20**, 41.

TABLE II.
Repeated Titrations of Anticoagulase of Control Sera.

| Serum | Anticoagulase titer | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 |
| 2 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 |
| 3 | 278 | 417 | 278 | 278 | 278 | 179 | 179 | 417 | 417 | 278 | 417 |
| 4 | 278 | 278 | 278 | 278 | 278 | 179 | 278 | 417 | 417 | 278 | 278 |
| A | 179 | 125 | 179 | 125 | 179 | — | 125 | — | — | — | — |
| B | 900 | 900 | 900 | 900 | 900 | 1400 | 900 | 900 | 900 | 900 | 900 |
| C | 278 | 278 | 417 | 278 | 417 | 417 | 278 | 278 | 278 | — | — |
| D | 417 | 278 | 417 | 278 | 417 | 417 | 417 | — | — | — | — |

Anticoagulase titers of sera 1, 2, 3, and 4 were determined on different days between 7/15/48 and 8/2/48.

Sera A, B, C, and D were used as controls between 8/26/48 and 1/29/49.

sera be employed in the antibody titration within 24 hours since within 48 hours small amounts of activator may again be detected.

The system used for measuring inhibitor to coagulase employs 0.5 ml of fibrinogen containing 1:1000 activator, whereas in the titration of coagulase, 1.0 ml of fibrinogen containing a dilution of 1:3500 activator is used. This procedure of increasing the activator concentration in the antibody titrations was employed so that technical variations in the amounts of coagulase or activator added, or residual amounts of activator remaining in the serum being tested, would not alter the end-point appreciably. The present test, as compared to that devised by Lominski and Roberts,[6] controls the concentration of activator in the fibrinogen and therefore more reproducible results should be obtained.

The stability of the various reagents used in this test is not definitely known. The fibrinogen solutions should be employed within a few hours of preparation. When stored at –20° or 4°C in sealed ampules, coagulase retains most of its activity over a period of a year or more, but repeated standardizations are best performed at monthly intervals. Tryptose broth is added as a diluent to the coagulase because it appears to stabilize its action so that results are reproducible. Activator, when stored in sealed ampules at –20°C or –4°C or at 4°C in rubber stoppered flasks for over a year, appeared to have lost little activity. When stored at room temperature, however, a loss of activity was observed.

*Summary.* A satisfactory serological test for the titration of anticoagulase in human and animal sera has been devised by controlling the various factors which enter into the coagulase-anticoagulase reaction.

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## Effect of Dibuline* on Nocturnal Gastric Secretion in Man. (17383)

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It has been shown[1-5] that dibuline (dibutyl urethane of dimethyl ethyl-B-hydroxyethyl ammonium sulfate, Merck) has a qualitative effect similar to that of atropine.

* Supplied by Dr. M. I. Grossman, University of Illinois, Chicago, Ill.

1 Swan, K. C., and White, N. G., *J. Pharm. and Exp. Therap.*, 1944, **80**, 285.

2 Featherstone, R. M., and White, N. G., *J. Pharm. and Exp. Therap.*, 1945, **84**, 105.

3 Peterson, C. G., and Peterson, D. R., *J. Pharm. and Exp. Therap.*, 1945, **84**, 236.

It possesses both a smooth muscle-inhibiting and an antiacetylcholine action. Marquardt, Case, Cummins, and Grossman[6] have reported a temporary decrease in the gastric secretory rate in dogs and humans given 10 mg subcutaneously; the effect was of short duration, the secretion returning to control levels within an hour. The purpose of this paper is to summarize a study of the effect of dibuline on the nocturnal gastric secretion of 14 patients with duodenal ulcer and one with functional bowel distress.

*Method of study.* The group consisted of 14 males and one female (14 with duodenal ulcer and one with functional bowel distress).

I. *The effect of a single injection* of dibuline was determined in 45 studies in fasting individuals. After a control period of 6 hours from 2:30 to 8:30 p.m., dibuline was given intramuscularly in a dose of 10 mg and its effect noted in the subsequent 4 hours. The gastric contents were aspirated continuously throughout the entire period of observation, the collection bottles being replaced at hourly intervals. The volume, concentration and total output of free hydrochloric acid were measured for each hour. A depressant effect on gastric secretion was considered significant if the reduction was greater than 25% and it was sustained for as long as 2 hours.

II. *Data on the total nocturnal gastric secretion* were obtained in a series of 15 experiments. The conditions of study were identical in all. All studies were made between 9:30 p.m. and 9:30 a.m. The gastric contents were aspirated continuously, the containers being replaced at hourly intervals. The volume, concentration of free hydrochloric acid, and total output of free hydrochloric of each hourly sample were determined by the usual method. Control hourly values for the 12-hour nocturnal gastric secretion were obtained for one night prior to the use of dibuline. During the second night, 10 mg of dibuline in 1.0 cc of water were given intramuscularly at 9:30 p.m., 1:30 a.m. and 4:30 a.m. The patients did not experience any pain at the site of the injection. No effect was considered significant unless a change of 25% or more was obtained.

TABLE I.
Effect of 10 mg Dibuline I.M. on Gastric Secretion in Man.

| | Vol., % | Conc. free HCl, % | Free HCl mg, % |
|---|---|---|---|
| Increase > 25% | 31 | 26 | 33 |
| No significant change | 43 | 34 | 27 |
| Decrease > 25% | 26 | 40 | 40 |

*Results. Effect of a single injection of dibuline* (Table I). The hourly volume was higher than the control values in 31% of the studies and did not change in 43%. A decrease of 25% or more from the control values was noted in 26% of the studies. Both the concentration and the output of free hydrochloric acid were lower in 40% of the studies. On the other hand, values greater than the control occurred during the administration of dibuline in 26 and 33%, respectively.

*Effect on the 12-hour nocturnal gastric secretion.* (Table II). In 3 individuals, the volume exceeded the control values. It was not affected significantly in 8 studies; lower volume was noted in 4 studies. In 3 of the latter, the concentration and output of free hydrochloric acid were diminished simultaneously. In 4 additional patients the concentration of free hydrochloric acid diminished although the volume of gastric secretion was unaffected. The total output of free hydrochloric acid (mg) was unchanged in 4 studies, considerably higher than the control values in 5, and lower in 6.

*Discussion.* The present results indicate that the effect of dibuline on gastric secretion in man is variable and similar to that obtained with atropine sulfate.[7] Both increases and decreases in secretion, as compared with the control period, were observed. The decreases particularly seem to be of sufficient magnitude to be of some significance. The recommended dose of dibuline (10

[4] Peterson, C. G., and Peterson, D. R., *Gastroenterology*, 1945, **5**, 169.

[5] Cummins, G. M., Marquardt, G. H., and Grossman, M. I., *Gastroenterology*, 1947, **8**, 205.

[6] Marquardt, G. H., Case, J. T., Cummins, G. M., and Grossman, M. I., *Am. J. Med. Sci.*, 1948, **216**, 203.

[7] Levin, E., Kirsner, J. B., and Palmer, W. L., to be published.

TABLE II.
Effect of Dibuline (10 mg I.M. at 9:30 p.m., 1:30 and 5:30 a.m.) on 12-Hour Nocturnal Gastric Secretion in Man.

| Case | Control | | | During Dibuline | | | % change | | |
|---|---|---|---|---|---|---|---|---|---|
| | Vol. (cc) | Free HCl (Cl units) | Free HCl (mg) | Vol. (cc) | Free HCl (Cl units) | Free HCl (mg) | Vol. | Free HCl (conc.) | Total output HCl |
| 1 | 435 | 75 | 1191 | 744 | 54 | 1456 | +71 | —28 | + |
| 2 | 998 | 89 | 3200 | 1456 | 79 | 4170 | +46 | — | +30 |
| 3 | 401 | 49 | 719 | 506 | 34 | 624 | +26 | —30 | — |
| 4 | 1188 | 42 | 1833 | 1047 | 69 | 2616 | — | +64 | +43 |
| 5 | 553 | 67 | 1352 | 671 | 76 | 1851 | + | + | +37 |
| 6 | 922 | 59 | 1988 | 1103 | 65 | 2593 | + | + | +30 |
| 7 | 583 | 60 | 1282 | 671 | 72 | 1761 | + | + | +37 |
| 8 | 1188 | 90 | 3892 | 1074 | 94 | 3690 | — | + | — |
| 9 | 1026 | 83 | 3103 | 1207 | 78 | 3416 | + | — | + |
| 10 | 598 | 56 | 1211 | 616 | 35 | 790 | + | —38 | —35 |
| 11 | 740 | 58 | 1556 | 622 | 39 | 885 | — | —33 | —43 |
| 12 | 1008 | 33 | 1208 | 757 | 14 | 387 | —25 | —58 | —68 |
| 13 | 1232 | 63 | 2802 | 726 | 33 | 878 | —41 | —48 | —69 |
| 14 | 1098 | 48 | 1908 | 591 | 33 | 491 | —55 | —31 | —74 |
| 15 | 572 | 42 | 875 | 214 | 68 | 526 | —63 | +64 | —40 |

mg) seems comparable to 1.0 to 2.0 mg of atropine. The decrease in gastric secretion, when it occurs, persists usually for 2 to 3 hours only. The repeated administration of the drug 4 hours after the initial dose is no more effective than the initial injection.

Dibuline is tolerated better than atropine. The parenteral injection of the latter in 1.0 mg doses every 4 hours, very frequently produces marked dryness of the mouth, blurring of vision, and tachycardia. Repeated injections of 10 mg of dibuline every 4 hours very seldom produce side effects; when they do occur, however, the symptoms are mild and of brief duration.

*Conclusions.* The effect of dibuline on gastric secretion is variable, transitory, and unpredictable. Further trial of the drug in the treatment of peptic ulcer seems to us unwarranted.

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## Possible Sources of the Androgenic Factor in Cow Manure. (17384)

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The bacterial flora of the stomach of a ruminant is known to synthesize a number of compounds: proteins from urea;[1] vitamin B complexes,[2,3] etc. It was felt desirable to determine changes in 17-ketosteroids on incubated feces.

Turner[4] finds an androgenic factor in fecal material of pregnant cattle and goats. Longwell and Gassner[5] report increased size of comb when chicks are fed dried fecal material as part of their diet. They have made some attempt at characterization of the compound but have not reached a definite conclusion as to its chemical nature.

It seems desirable to investigate two pos-

1 Smith, J. A. B., and Baker, F., *J. Biochem.*, 1944, **38**, 496.

2 Hunt, C. H., Burroughs, E. W., Bethke, R. M., Schalk, A. F., and Gerlaugh, Paul, *J. Nutrition*, 1943, **25**, 207.

3 Burkholder, Paul R., and McVeigh, Ilda, *Proc. Nat. Acad. Sci. U. S.*, 1942, **28**, 285.

4 Turner, C. W., *J. Dairy Science*, 1947, **30**, 1.

5 Longwell, Bernard B., and Gassner, F. X., *Fed. Proc.*, 1947, **6**, 272.

sible sources of the androgens found in the feces of pregnant cattle and to study the properties of the material extracted: first, its production by bacterial flora upon incubation of fecal material; and secondly, its presence in demonstrable amounts in the bile of pregnant cows. Immature rats, ovariectomized female rats and single comb White Leghorn baby chicks, both sexes, served as experimental subjects.

*Experimental. Collection and disposition of samples of feces.* The cow manure, free of urine, was collected within 2 hours after dropping from a pregnant cow of 7½ months duration. The sample was thoroughly mixed and divided into 3 portions which were placed in sterilized fermentation jars. Sample No. 1 weighed 3333 g; No. 2, 3379 g; No. 3, 3751 g. Sample No. 1 was immediately covered with absolute alcohol in such amounts that with the water present, the mixture was about 70% alcohol. The untreated samples No. 2 and No. 3 were placed in an incubator at 37.5°C, the former remaining for one week, and the latter for 2 weeks.

*Extraction of feces.* The feces, which was placed in absolute alcohol as indicated above, was extracted in the cold 3 times with agitation, using calculated amount of 95% alcohol to give about 70% concentration. The combined extracts were allowed to stand overnight, centrifuged and decanted. This solution was evaporated to dryness under reduced pressure to a small volume. It was then boiled with norite and filtered through super sal to remove colored matter. The alcohol solution was evaporated to dryness under reduced pressure and this procedure repeated until all the water was removed. The residue was taken up in alcohol so that 1 ml contained the equivalent of 15 g of fresh manure. After their respective periods of incubation, samples No. 2 and No. 3 were treated in a like manner. All were very dark in color, which boiling with norite and filtering through super sal did not remove.

Such high dilutions had to be made in order to be able to apply the Zimmermann test, that the following further attempt was made to remove the chromogenic material. An aliquot of each sample was evaporated to dryness under diminished pressure, taken up in water and extracted 3 times with carbon tetrachloride. These were then washed twice with 2N NaOH, and once with 10 cc of Lycopon (1N NaOH with 10 g of sodium hydrosulfite). The $CCl_4$ was evaporated to dryness under diminished pressure and the residue dissolved in alcohol. Samples No. 1 and No. 3 still were a dark brown color and No. 2 was a bright yellow color. Later tests with the Zimmermann test indicate that for some reason No. 2 gave no definite absorption band at 508 mμ.

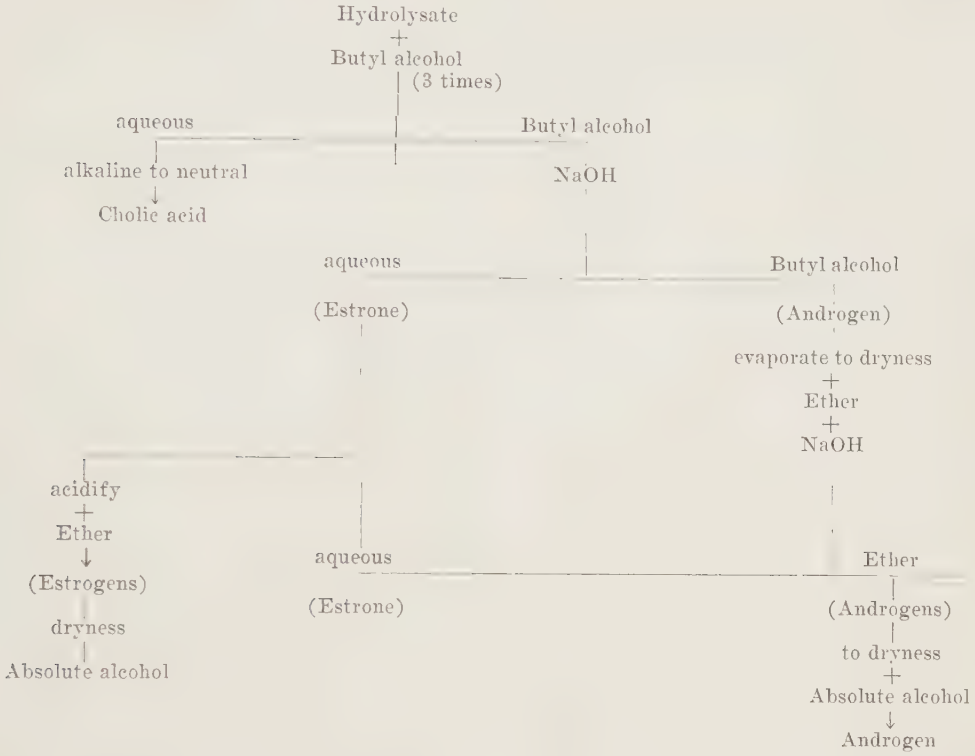
Following the alcoholic extraction of the feces, some of the extract from sample No. 1 was then evaporated to dryness and extracted 3 times with ether. The ether was evaporated to dryness under diminished pressure, and the residue dissolved in absolute alcohol. This will be referred to as sample No. 4.

*Extraction of bile.* Bile was obtained from pregnant cattle of 3-4 months duration. To it was added one-fourth its volume of concentrated hydrochloric acid, and the mixture boiled for 15 minutes. The hydrolysate was then treated as shown on following page.

*Test for androgenic factor using baby chicks; application of extract of feces.* Two-day-old single comb White Leghorn chicks of both sexes were used in the test. The extract, equivalent to 3 g fresh manure, was applied to the comb each day for about 4 weeks. This was done only on the fresh extract and not on incubated fecal material in order to demonstrate the presence of androgens in the sample under consideration. Increased comb growth was noted in both male and female chicks, but it is not of mathematical significance.

*Application of extract of bile.* The extract of bile was applied topically exactly as described for application of fecal extract. An equivalent of 3 ml of fresh bile was applied daily on each chick's comb. An increase in comb growth of both male and female chicks was demonstrable.

*Spectrophotometric studies.* Samples before and after attempt to remove chromogens were equilibrated and tested for 17-ketosteroids by the Zimmermann reaction. K-values (where $K = \log I_o/I$) for these before




and after the removal of the chromogens are shown below at 508 mμ for the original sample (No. 1) and the 2 which were incubated for one and 2 weeks (Nos. 2 and 3, respectively).

The K-value obtained is a comparative measure of the quantity of the 17-ketosteroids present. Since there was no increase in the K-value, it is conclusive that no increase of 17-ketosteroids was experienced on incubation and that therefore no increase of androgens was evident upon incubation as carried out. Since there was no increase in 17-ketosteroids, no biological androgenic assay was considered necessary to prove that the bacterial flora of the feces was not responsible for the increased androgenic content of pregnant cow manure.

TABLE I.

| Sample | Col. I Before | Col. II After |
|---|---|---|
| 1 | 1.524 | 1.533 |
| 2 | 1.164 | no peak |
| 3 | 1.255 | 1.322 |

*Results and conclusions.* 1. The Zimmermann test showed that there was no significant difference in the amount of 17-ketosteroids before and after incubation periods up to 2 weeks. Since the material tested in column II has been extracted thoroughly with 2N NaOH, little estrogenic substance can be present. Hence the figures obtained are believed to indicate androgens.

2. It is therefore believed that the bacterial flora of the ruminant stomach is not concerned with the increased androgenic content of the feces of a pregnant cow. If any changes are obtained, it appears that there is a loss of androgens during incubation, or a loss by the second treatment.

3. The bile from pregnant cows showed a demonstrable amount of an androgenic substance.

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# Alkaline Phosphatase in the Mouse Thyroid Following Testosterone Propionate, Thiouracil and Thyroglobulin.* (17385)

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The BMR of patients is increased by testosterone propionate and suggests that this androgen might influence the thyroid gland.[1] In animals, testosterone propionate has been reported to increase mouse thyroid weight[2] and to increase mitotic figures in rat thyroids.[3] It should be noted, however, that other investigators have failed to find an effect of testosterone on thyroid weight in mice[4] and rats[5] as well as in rats fed thiouracil.[5,6] Since weight was the essential criterion of effect and this factor is variable, it seemed more plausible to reinvestigate this problem on a histochemical basis and the alkaline glycerophosphatase technic was chosen.[7] This enzyme was not found in the rat thyroid in initial studies[7–9] but Dempsey and Singer[10] obtained positive results with longer incubation times. Alkaline phosphatase of the mouse thyroid has not been described but our preliminary studies revealed its presence making possible the androgen study in this species.

In addition to the effect of testosterone propionate on the normal thyroid it was of interest to determine whether the androgen would influence a thyroid gland altered by thiouracil or thyroglobulin feeding. Changes in physiological state may alter the alkaline phosphatase concentration. For example, stress induced by cold reduced enzyme concentrations[11] but no definite conclusions were drawn from thyroids of 2 rats fed thiouracil.[10] Kroon[12] observed an increased alkaline phosphatase in the distended thyroid blood vessels following thiouracil feeding to rats. Hypophysectomy did not effectively alter the alkaline phosphatase of the rat thyroid follicle.[13] The influence of thiouracil or thyroglobulin feeding alone on the mouse thyroid is presented.

Twenty-two day old Swiss albino female mice were used and maintained on Purina Fox Chow. Groups of 7-11 animals were given testosterone propionate (Perandren, Ciba†) in a single injection of 0.05 mg, 0.10 mg, 0.20 mg, or 0.50 mg when 32 days old. One group of 5 animals received a single injection of 2.5 mg of the androgen. The mice were sacrificed 7 days later. Control mice were autopsied at 32 and 39 days of age.

To study the influence of hypo- and hyperthyroidism alone and with testosterone propionate, 0.25% thiouracil or 0.1% thyroglobulin (Proloid) was incorporated in the Fox Chow diet. The drug feeding was started

* This investigation was done under contract with the Office of Naval Research, Navy Department.

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11 Dempsey, E. W., *Ann. of N.Y. Acad. Sci.*, 1949, **50**, 336.

12 Kroon, D. B., *Acta Endocrinology*, 1949, **2**, 227.

13 Dempsey, E. W., Greep, R. O., Deane, H. W., *Endocrinology*, 1949, **44**, 88.

† Testosterone propionate (Perandren, Ciba) was supplied by Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, N. J., Thiouracil (Deravet) was supplied by Dr. Mark Welsh, Lederle Laboratories, Pearl River, N. Y., and Thyroglobulin (Proloid) was provided by Dr. Robert Kroc, The Maltine Company, Morris Plains, N. J.

TABLE I.
Effect of a Single Injection of Testosterone Propionate on Alkaline Phosphatase Content of Mouse Thyroid Gland.

| Testosterone propionate dosage, mg | Mice fed fox chow only | Mice fed fox chow plus 0.25% thiouracil | Mice fed fox chow plus 0.10% thyroglobulin |
|---|---|---|---|
| 0 | + | 0 | + |
| .05 | + | 0 | + |
| .10 | + | 0 | + |
| .20 | + | 0 | + |
| .50 | ++ | 0 | + |
| 2.50 | +++ | | |

0 = An overall negative phosphatase content.
+ = Nuclear and slight cytoplasmic content.
++ = Nuclear and moderate cytoplasmic content.
+++ = Fairly heavy content in the nuclei and cytoplasm of the acinar cells; slight content in the nuclei of the interfollicular cells.

at 22 days of age with autopsy at 32 and 39 days of age. The androgen, when administered, was injected on the 32nd day of age and permitted to act over 7 days thus a preliminary drug feeding period of 10 days preceded injection and in all cases drug feeding was continued after hormone administration.

Mice were killed by cervical dislocation. The right thyroid fixed in cold 80% alcohol and the left thyroid in Bouin's fluid. Alkaline glycerophosphatase was checked on sections cut at 7 $\mu$ and prepared by the Gomori technic[14] with the addition of a 2% $MgSO_4$ solution to the incubation mixture to enhance the stain. Incubation was carried out at 37° for 7 hours at pH 9.2-9.4. The Bouin's fixed material was sectioned at 10 $\mu$ and stained with hematoxylin and eosin.

The thyroid gland of immature female mice has alkaline glycerophosphatase essentially concentrated in the nuclei of the follicular cells with a tinge appearing in the cytoplasm. This condition was not altered by testosterone propionate until a dosage of 0.5 mg was reached when, however, the enzyme concentration exhibited an increase in the cytoplasm in all 8 mice studied (Table I). This proved a critical level, but an even more striking effect on the cytoplasmic phosphatase could be seen with a 2.5 mg dosage. Routine sections stained with hematoxylin and eosin failed to reveal an androgen effect.

Thiouracil feeding resulted in the anticipated hypertrophy of the thyroid epithelium and loss of colloid. Alkaline phosphatase was essentially lacking as the enzyme was not present in the cytoplasm and was reduced or absent in the nucleus of the follicular cells. Only the endothelial lining of the blood vessels contained alkaline phosphatase to any degree. Administration of testosterone propionate failed to alter this essentially negative enzyme picture even at the 0.5 mg dose which was effective in the normal mouse (Table I). The general histology also was not altered.

Following thyroglobulin feeding the thyroid histology was that of an atrophic gland as the follicular epithelium was now low cuboidal or squamous. Alkaline phosphatase was definitely present in the nucleus and the cytoplasmic staining concentrated so that the follicular epithelium frequently appeared as a line of alkaline phosphatase. Essentially the same histology has been described for the rat thyroid following hypophysectomy.[13] Testosterone propionate in the dosages used failed to alter the alkaline phosphatase histology and provided no evidence of an effect in our routine preparations (Table I).

Increased activity of the thyroid gland is correlated with a loss in alkaline phosphatase whether induced by stress or thiouracil feeding. Furthermore, pituitary activation of the thyroid or disuse atrophy due to thyroid administration plays a primary role in the control of enzyme concentration since testosterone propionate can not override their effects. Nevertheless, in a normal female mouse, androgen can increase the alkaline

[14] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

phosphatase concentration suggesting the use of histochemical technics to evaluate steroid hormone actions on the thyroid and at the same time emphasizing the importance of the physiological state of the end organ and its effect on anticipated results.

*Summary.* Testosterone propionate definitely increased the cytoplasmic alkaline phosphatase in the follicular cells of the mouse thyroid when dosages of 0.5 mg or more were used. Thiouracil (0.25%) induced marked hyperplasia in 17 days and virtually eliminated the alkaline phosphatase in the follicular epithelium whereas thyroglobulin caused a follicular cell atrophy without loss of the enzyme. Testosterone propionate did not influence the thyroid alkaline phosphatase in the presence of thiouracil or thyroglobulin.

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## Lysozyme Titres in Regional Enteritis, Miscellaneous Tissues, Microorganisms, and Excreta.* (17386)

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(Introduced by Karl Meyer.)

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The apparent increase in the lysozyme content of the stools in regional enteritis has already been reported.[1] At that time the proposition that lysozyme is a local initiating agent in the pathogenesis of this disease (as well as in chronic ulcerative colitis) was advanced. The circumstantial evidence supporting this conclusion was reported there and in an accompanying article discussing the probable etiologic role of lysozyme in peptic ulcer.[2]

Only 6 cases of regional enteritis were reported at that time; it therefore appears desirable to supplement this evidence with that subsequently obtained.

The values in the additional cases studied appear in the table. All were regional ileitis with involvement of varying lengths of bowel. The disease process ceased at the ileo-cecal valve in every instance. The assays were done by the viscosimetric technic of Meyer.[3]

The results were markedly uniform except for 2 low titres and one quite high one. The former were from constipated patients and the latter individual had marked diarrhea. It has already been noted that the rate of fecal expulsion influences the stool titre, particularly when the disease process does not extend to the descending colon. This depends upon the gradual inactivation of fecal lysozyme re-

TABLE I.

| Case | Lysozyme concentration in units/g of stool (wet wt) | Lysozyme output per day in units |
|---|---|---|
| 1 | 17.8 | 1,727 |
| 2 | 3.6 | — |
| 3 | 14.8 | 17,538 |
| 4 | 18.2 | 1,170 |
| 5 | 32.9 | 3,258 |
| 6 | 21.3 | — |
| 7 | 1.7 | 1,120 |
| 8 | 22.4 | 2,910 |
| 9 | 15.4 | — |
| 10 | 22.2 | — |
| 11 | 14.3 | — |
| 12 | 17.0 | — |
| 13 | 20.4 | — |
| 14 | 55.0 | 15,817 |
| Means | 19.8 | 6,220 |
| Avg stool lysozyme values in normal individuals (1) | 2.7 | 158 |
| Avg stool lysozyme values in purged individuals (1) | 1.6 | 1,064 |
| Avg stool lysozyme values in chronic ulcerative colitis (1) | 73.6 | 26,568 |

* Supported in part by the Research Grants Division of the U. S. Public Health Service.

1 Meyer, K., Gelhorn, A., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am. J. Med.*, 1948, **5**, 496.

2 Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am. J. Med.*, 1948, **5**, 482.

3 Meyer, K., and Hahnel, E., *J. Biol. Chem.*, 1946, **163**, 733.

TABLE II.

| Specimen | Lysozyme titer in units/g or cc |
|---|---|
| 1. Human fetal tissue (13 wk) | |
| a. whole intestine | 39.8 |
| b. thigh muscles | 33.4 |
| c. lung | 13.3 |
| d. iliac bone | 25.0 |
| e. skin | 19 |
| f. placenta | 100 |
| g. " | 100 |
| 2. Dog amniotic fluid | 0 |
| 3. Two full term placentas (human) | 22.9 61.6 |
| 4. Endometrium (human) | |
| a. 14th day of cycle | 8.1 |
| b. 26th day of cycle | 14.8 |
| c. 3rd day menstrual discharge | 27.8 |
| d. bloody discharge following 4 months miscarriage | 18.5 |
| 5. Nephrotic urine (normal urine—less than 1) | 6 |
| 6. Pus | |
| a. from acute staphylococcal abscess over elbow joint | 237 |
| b. from chronic *Clostridium welchii* peritoneal abscess | 80 |
| c. from chronically infected pilonidal abscess (anerobic strep.) | 280 |
| 7. Mass cultures of intestinal flora in two patients with chronic ulcerative colitis | 0 ; 0 |
| 8. Stool from cases of acute gastroenteritis due to | |
| a. *Salmonella typhimurium* | 6.3 |
| b. Paratyphoid B | 11.5 |
| 9. Paratyphoid B culture | 0 |
| 10. Acute non-specific infantile diarrhea-stool | |
| a. 1st day of disease | 125 |
| b. 7th day of disease | 5 |
| 11. Dog gastric juice | |
| a. under anesthesia | 2.3; 1.6; 5.5 |
| b. conscious (acidity 0/4, 0/28) | 5.3; 21.3 |
| 12. Enterocystoma fluid (small bowel mesentery) | 125 |

maining in the gut over time. It is considered that this explains the two-way variation in these 3 cases.

A comparison of these values to those previously reported in chronic ulcerative colitis, normal subjects, and normal subjects with purging shows the definite elevation of lysozyme concentration in regional enteritis. This confirms the results in the 6 cases already reported.

In addition, 3 samples of mucosa from resected segments of involved ileum were assayed. They contained 12, 66.6 and 36.2 units/g of mucosa respectively.

These data confirm the previously published impression that regional enteritis is a disease characterized by high lysozyme concentrations in the stool and in the mucosa. The mucosal assays suggest that the pathogenesis of this entity is identical with that of chronic ulcerative colitis, and that the pathological differences between them are caused by the variable response of any one tissue to injury. The cause of the localization of increased mucosal lysozyme in any one gut segment is not known.

The lysozyme titres associated with the normal gastrointestinal tract, peptic ulcer,

chronic ulcerative colitis, body fluids and secretions, hyaline cartilage, and granulation tissue have previously been reported in detail.[1-4] It is the purpose here to record additional noteworthy assays.

The results are shown in Table II.

Many of these findings cannot be discussed because of lack of knowledge concerning the physiologic role of lysozyme. Some determinations, however, deserve comment because of their relation to inflammatory intestinal disease. For example, high stool lysozyme titres apparently are not a necessary corollary to acute specific gastrointestinal inflammation (see 8 in table). On the other hand, a previously reported[1] case of acute amebic dysentery had a stool titre of 38 units/g (upper limit of normal stool lysozyme concentration is about 9 units/g). Likewise, one case of acute nonspecific diarrhea in an infant had a high stool titre which fell rapidly to normal over 7 days (see 10 in table). This case had no gross or occult blood in the stool.

The high lysozyme content of one of the 2 canine gastric juices obtained by Levine tube aspiration without anesthesia (? psychic stimulation) is of interest because a great many determinations on dog gastric juice, gastrointestinal mucosa, and stools in the absence of exciting or disturbing factors have been uniformly less than 6 units/g or cc.

The absence of any lysozyme in the mass cultures from chronic ulcerative colitis cases (see 7 in table) confirms the impression that intestinal lysozyme is not bacterial in origin.

*Summary.* The stools of 14 regional enteritis cases possessed a mean lysozyme content of 19.8 units per gram of stool (wet weight) and a mean daily output of 6,220 units. This is 7.34 times the mean lysozyme content of normal stools and 39.4 times the mean daily output in the stools of normal persons. These data, together with the experimental production by lysozyme of ulcerations in the canine alimentary tract,[2] indicate that lysozyme is an etiologic agent in the pathogenesis of regional enteritis.

A survey of various microorganisms, tissues, and excreta revealed generally high titres in fetal tissues and placenta, moderately high levels in endometrium with a suggestion of cyclic variation, and very large amounts in pus and in fluid from an enterocystoma. Low titres were found in the stools of two acute specific dysenteries and in canine gastric juice, with no measurable amounts in mass cultures from two chronic ulcerative colitis cases and one paratyphoid B culture. A case of acute infantile diarrhea exhibited a very high titre on the day of onset in the absence of organic pathology or specific infection, falling rapidly to normal with recovery.

It is felt that the generally high titres of fetal tissue suggest the possibility of an important role for lysozyme in the chemistry of actively growing normal tissue. The absence of lysozyme in the colitis mass stool cultures substantiates the conclusion that intestinal lysozyme is not bacterial in origin; and the moderately low levels in the specific dysentery stools indicate that acute colitis is not necessarily accompanied by a high stool lysozyme titre.

We acknowledge the generous advice of Dr. Karl Meyer and Dr. John S. Lockwood.

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[4] Prudden, J. F., Lane, N., and Meyer, K., to be published.

# Viral Agglutination of Saponin-Lysed Chicken Erythrocytes. (17387)

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The results of recent studies concerning the mechanism of hemagglutination by viruses have brought out certain facts pertaining to the presence, on or within the surface structure of agglutinable erythrocytes, of receptor-like substances which can be removed by appropriate procedures. It has been established,[1] for example, that treatment of human group O erythrocytes with a filtrate of a broth culture of *Vibrio comma* results in a suspension of cells which have lost their capacity to adsorb or be agglutinated by influenza virus. It appears that substances with such receptor-destroying properties produce no drastic alteration of the red cell architecture. This is evidenced by the fact that treatment of a suspension of chicken erythrocytes with influenza virus renders the cells receptor-free without producing any obvious change in the appearance of the suspension or in the morphology of the component cells. On the other hand, normal chicken erythrocytes which have been lysed, or partially lysed, by mechanical trauma, water, or dilute saponin retain the ability to adsorb[2,3] and be agglutinated[4] by influenza virus. A consideration of these facts raised the question as to whether a hemolytic agent such as saponin has no effect on the surface receptors or whether additional "internal" or "nuclear" receptors are exposed by the lytic process. The present paper provides information in support of the former possibility in that it is shown that chicken erythrocytes rendered inagglutinable by treatment with influenza virus are not agglutinated by the virus after lysis in dilute saponin. Experiments in this connection were prefaced by a quantitative study of the agglutinability of saponin-lysed normal erythrocytes by the virus. Methods were developed for the preparation and use of suspensions of lysed cells in agglutination and inhibition titrations.

*Preparation of suspensions of lysed cells.* It was observed that if chicken erythrocytes were lysed with saponin by appropriate procedures, resulting suspensions were homogeneous, colorless, and opaque. Examination in a wet mount by ordinary or phase microscopy revealed the presence of particles which superficially resembled erythrocyte nuclei. The periphery, however, was not discrete and had a coarsely granular appearance. A considerable amount of amorphous extranuclear material, apparently stroma, was seen in heavily stained films.

Lysed cells can be used in agglutination (CCA) and –inhibition (CCAI) titrations, set up according to the Salk method,[5] only if the suspensions are prepared properly. It was found that almost every step in the procedure is critical. The ratio of number of cells to volume of saponin is of some importance, but of more importance are the concentration of saponin and the time the cells are allowed to remain in the saponin before centrifugation and washing. The lysing procedure that was adopted is as follows: one ml of adequately washed and packed chicken erythrocytes are added to 100 ml of 0.125% saponin. The cells are allowed to lyse for 6 minutes with constant shaking by hand. The suspensions are centrifuged at low speed (1800 R.P.M.), and are washed twice and resuspended in 0.9% NaCl.

Suspensions of lysed cells prepared in this manner were used in the early phases of the work. CCA and CCAI tests were not easy to read, however, in that the pellets and agglutination patterns were colorless. This situation was remedied by the addition of 2-3 ml of a 0.5% aqueous methyl green solu-

1 Burnet, F. M., *Brit. J. Exp. Path.*, 1946, **27**, 228.

2 Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

3 Lajmanovich, S., and Mittleman, N., *Rev. Soc. Argentina Biol.*, 1946, **22**, 339.

4 Carlisle, H. N., and Elrod, R. P., *Soc. Am. Bact. Proc. Meetings*, 1949, **2**, 92.

5 Salk, M. E., *J. Immunol.*, 1944, **49**, 87.

tion to the 100 ml of saponin used for lysis. In this manner, the erythrocytes were lysed and stained simultaneously. The use of methyl green stained suspensions definitely facilitated the reading of agglutination and inhibition titrations. Pellets were blue-green in color and the supernatant was colorless. The staining procedure did not affect the agglutinability of the particles. It appears that methyl green does not have the sensitizing effect in this reaction that it does in certain other agglutination systems.[6]

*Use of lysed cells in CCA and CCAI tests.* Suspensions of lysed erythrocytes were used to measure the CCA capacity of allantoic fluids containing the PR8 and Lee strains of influenza virus. Antisera against these agents prepared in chickens and rabbits were utilized in the CCAI titrations. All tests were set up according to the method of Salk.[5] When methyl green stained suspensions were used, pellets, rings and positive patterns were as discrete, and end-points were as definite and readable as in parallel control titrations in which normal erythrocytes were used. However, since the particles in the suspensions of lysed cells were smaller than erythrocytes, they sedimented more slowly, and a longer time was required for good pellet formation. All tests were read after storage overnight at approximately 4°C.

The end-points of agglutination titrations were governed by the concentrations of the suspension of lysed cells. As the concentration was decreased, the agglutination titer of virus preparations increased. Since this relationship between concentration and titer existed, suspensions of lysed cells could be adjusted with saline so that they yielded the same end-points as were obtained in parallel titrations of virus against normal erythrocytes. And, if such adjusted suspensions were used in inhibition titrations, end-points were the same as when erythrocytes were used. It was determined that suspensions of lysed cells which gave these similar titers contained 2.5 times as many particles per unit volume as did the 0.25% erythrocyte suspension used in the control tests. The significance of this observation is being considered and will be discussed in a later communication.

*Experiments with receptor-free erythrocytes.* From the results obtained with normal erythrocytes it appeared that saponin had no effect on the receptors concerned in the agglutination of the cells by influenza virus. The possibility existed, however, that the surface receptors were destroyed and that additional receptors located within the cell were exposed by the lytic process. In view of this contingency, it was decided to investigate the agglutinability of suspensions of lysed cells prepared from chicken erythrocytes which had been rendered inagglutinable by treatment with virus. Receptor-free cells were prepared by the following method: One ml of adequately washed and packed erythrocytes was added to 9 ml of undiluted PR8 allantoic fluid (Salk titer, 2560). The virus-cell suspension was placed at 37°C for 6 hours, and was shaken gently every 30 minutes. The cells were then washed twice with 0.9% NaCl and packed to the original volume, one ml. The amount of hemolysis and change in color which occurred during the incubation period were negligible and did not exceed that observed in a similarly incubated 10% suspension of normal cells in saline. Receptor-free cells prepared in the above manner were not agglutinated by the virus. When used in Salk titrations, the pellets obtained in tubes containing virus were as well-formed as those in either the treated cell-saline or the normal cell-saline control tubes.

Virus-treated erythrocytes were lysed with saponin according to the method described previously. The resulting suspensions were indistinguishable grossly and microscopically from suspensions of lysed normal cells. They were not, however, agglutinable by influenza virus. Table I summarizes the results of one of three identical experiments in this connection.

*Comment and conclusions.* From these experiments it would seem that saponin has no effect on the receptors responsible for the agglutination of chicken erythrocytes by influenza virus. Internal receptors, if such exist, are not exposed during the lytic process. The

[6] Berger, F. M., *Brit. J. Exp. Path.*, 1943, **24**, 252.

TABLE I.
Agglutinability of Normal and Receptor-Free Erythrocytes Before and After Saponin Lysis.

| | Virus dilutions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10 | 100 | 200 | 400 | 800 | 1600 | 3200 | Saline |
| Normal, lysed | ++* | ++ | ++ | ++ | ++ | +R | RR | PP |
| Receptor-free, lysed | PP | PP | PP | PP | PP | PP | PP | PP |
| Normal, not lysed | ++ | ++ | ++ | ++ | ++ | ++ | PR | PP |
| Receptor-free, not lysed | PP | PP | PP | PP | PP | PP | PP | PP |

* Duplicate tubes.
\+ Complete agglutination.
R—Ring, partial agglutination.
P—Pellet, no agglutination.

agglutinability of saponin-lysed cells by the virus is, in all probability, due to unaltered surface receptors and not to any substance associated with the nucleus. This interpretation is valid, however, only if it can be assumed that virus particles do not penetrate to the interior of the erythrocytes during treatment with the virus. That such a process could occur seems unlikely in view of present knowledge concerning the interaction between red cell and virus.

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## The Tinctoral Demonstration of a Glycoprotein in Whipple's Disease. (17388)

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The etiology and pathogenesis of Whipple's Disease (lipodystrophy intestinalis), a clinical syndrome similar to sprue, has been a subject of speculation since its original description.[1] As its synonym implies, it is believed by most observers to represent an obscure disturbance of fat metabolism.

Black-Schaffer, Hendrix and Handler[2] reported a study of 4 cases which led them to the following conclusion: The disease, in contrast to sprue, may be readily recognized, anatomically, by non-lipid macrophagocytosis in the lamina propria of the small intestine and occasionally the proximal colon, lipogranulomatosis of the mesenteric lymph nodes, absence of significant evidence of chylous obstruction; and clinically, poor fat, glucose and probably protein absorption and the absence of macrocytic anemia.

The characteristic intestinal lesion is a crowding of the lamina propria by macrophages containing an isotopic refractile substance which Whipple found unstainable with osmic acid. This observation has been repeatedly overlooked in the literature, almost all authors assuming a lipid nature for this curious substance. The study of Black-Schaffer, Hendrix and Handler confirmed Whipple's observation. In 3 cases* the phagocytosed material did not stain with Sudan IV, and 2† were likewise negative with Nile blue sulphate as well as osmic acid. Chemical analysis of the intestinal mucosa of two cases revealed no increase, over normal controls, of the lipid content.

The characteristic enlarged, cystic, fat-filled mesenteric lymph nodes (lipogranulomatosis) are so prominent that they have dominated the

1 Whipple, G. H., *Johns Hopkins Hosp. Bull.*, 1907, **18**, 382.

2 Black-Schaffer, B., Hendrix, J. P., Handler, P., *Am. J. Path.*, 1948, **24**, 677.

* No suitable material was available from one case.

† No suitable material was available from 2 cases.

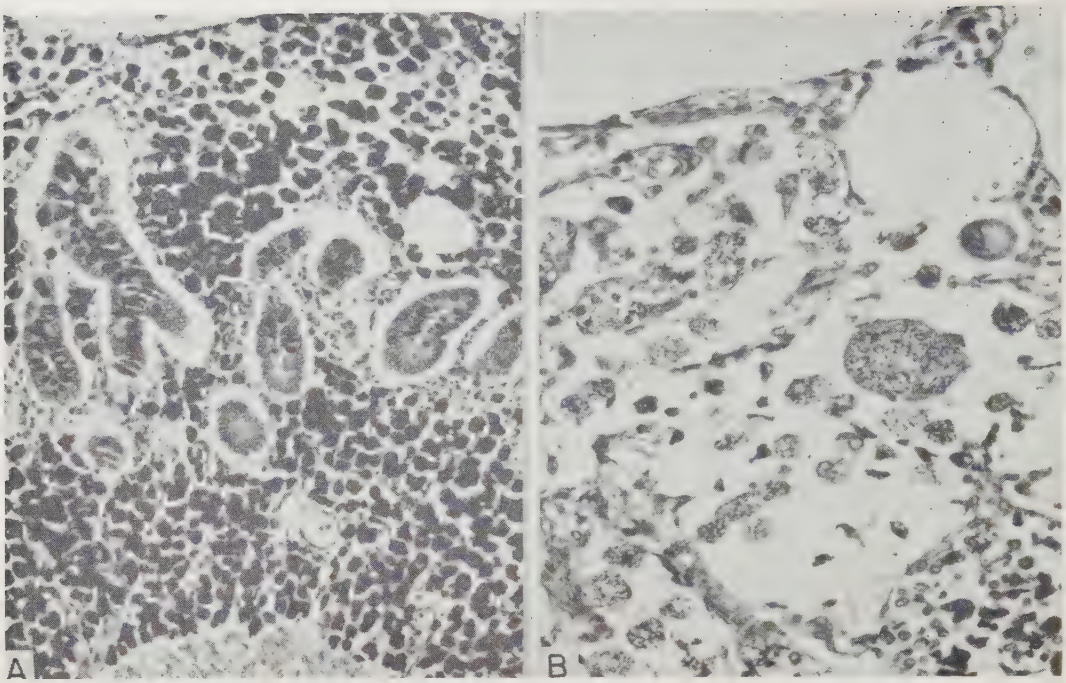


FIG. 1.

A. The mucosa of the small intestine in Whipple's disease occupied by macrophages filled with glycoprotein, stained by MacManus' modification of Schiff's periodic acid stain. The mucin of the "goblet cells" in the glands shows identical tinctoral properties.

B. Macrophages and giant cells in a mesenteric lymph node of Whipple's disease, revealing the presence of glycoprotein granules (in the photograph black) and lipid (colorless vacuoles). In the lower right corner are macrophages replete with glycoprotein and identical to those pictured in A.

approach to the problem.

Careful examination of the nodes revealed,[2] adjacent to sudanophilic macrophages, others containing sudanophobic substance similar in all respects to that described in the intestine. As a consequence of the pathologic anatomy, the histochemistry and the chemical analyses, a pathogenesis of the lesions was proposed.[2]

The present report is occasioned by the identification of the unknown phagotaxic substance as a glycoprotein. When sections of the intestines of 4 cases were treated with Schiff's periodic acid stain[3,4] the phagocytosed material in the mucosa stained deep scarlet (Fig. 1).

The lymph nodes of 3‡ of the cases confirmed the studies[2] of the fat-stained tissues. Many macrophages, in fact, surprisingly many, color a brilliant scarlet. Most of the giant cells surrounding the large masses of fat contain the same red substance (Fig. 2). Frozen sections treated with periodic acid and Sudan IV demonstrate lipid and polysaccharide side by side within the same macrophages. There is, however, no coalescing of the glycoprotein material to form amorphous masses as does the fat.

The periodic acid stain depends upon the oxidation of a 1-2 glycol bond in a polysaccharide forming polyaldehydes which thereupon take up the fuchsin of Fuelgin's reagent.

The polysaccharide containing material is insoluble in water, being present in apparently undiminished amounts after as long as 13 years in aqueous Kaiserling solution. Best's carmine stain[5] for glycogen is uniformly nega-

[3] McManus, J. F. A., *Stain Technology*, 1948, **23**, 99.

[4] Hotchkiss, R. D., *Arch. Biochem.*, 1948, **16**, 131.

‡ Material is unavailable from one case.

tive. On the other hand the mucicarmine stain[5] for mucus lends a faint tint to the phagocytosed substance. This, plus its insolubility in water—properties akin to those of the glycoprotein mucin which also stains scarlet to red with periodic acid—indicates that the polysaccharide is bound to a protein, thus constituting a glycoprotein.

The diminished fat, glucose, and possibly protein absorption by the small intestine in Whipple's disease indicates that its etiology resides in a disturbance of function of the intestinal epithelium. It is probably this defect which permits absorption of the unusual glycoprotein which may or may not be related to the mucin discharged into the enteric lumen by the intestine itself. At any rate, the presence of this readily demonstrated glycoprotein in the intestinal mucosa and mesenteric lymph nodes, indicates that Whipple's disease is more than an obscure defect in fat metabolism and is certainly not the result, as is commonly suggested, of a block of the mesenteric lymphatics. Thus the name lipodystrophy intestinalis, first proposed by Whipple and currently in use, would seem to be inappropriate. It would appear desirable to return to the eponym "Whipple's disease" until a name denoting the nature, rather than a complication, of the disease is forthcoming.

5 Mallory, F. B., Pathological Technic, W. B. Saunders Co., Philadelphia, 1938.

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## Inhibitory Effect of Cow's Milk on Influenza Virus Hemagglutination.* (17389)

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The development of theories on the mechanism of infection by influenza viruses has received considerable encouragement in the past few years from studies of the interaction between these viruses and inhibitors of virus hemagglutination found in various mammalian and avian fluids.[1-7] For some lines of inquiry it is desirable to compare the properties of several fluids, as well as those of the inhibitors isolated from them. It is of interest, therefore, that cow's milk, a fluid readily available in considerable quantity, exerts an inhibitory effect on virus hemagglutination. Some aspects of this inhibition phenomenon are described in the present report.

*Materials and methods.* Fresh raw milk was obtained from individual cows or as a pool from a commercial distributor and was skimmed by centrifugation in the cold room in the laboratory. The cream, which carried an insignificant part of the total inhibitory activity, was lifted off with a spatula and discarded. If necessary, the milk was recentrifuged to remove residual cream or sediment. Raw skim milk (RSM), less than one day old, was used for most of the experiments; if intended for use after one day, the milk was preserved with 1:5,000 Merthiolate (Lilly), which was found not to affect the inhibitory activity.

* This work was supported by the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C., and by a research grant from the National Cancer Institute, U. S. Public Health Service.

1 Burnet, F. M., *Lancet*, 1948, **254**, 7.

2 Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301, 315.

3 Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 442.

4 Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, **26**, 403.

5 Svedmyr, A., *Brit. J. Exp. Path.*, 1948, **29**, 295, 309.

6 Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **88**, 463.

7 Francis, T., Jr., and Minuse, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 291.

TABLE I.
Inhibitory Activity of Raw and Pasteurized Skim Milk Against Hemagglutination by Heated Swine Influenza Virus.

| Cow | Type of milk | Titer |
|---|---|---|
| 1 | Raw | 4100 |
| 2 | ,, | 5100 |
| 3 | ,, | 4100 |
| 4 | ,, | 6400 |
| 5 | ,, | 2600 |
| 6 | ,, | 6400 |
| 7 | ,, | 6400 |
| 8 | ,, | 5400 |
| Commercial pool | ,, | 2600 |
| ,, ,, | Pasteurized* | 460† |

* 30 min. at about 71°C by a vat process.
† Conventional endpoint. The slope of the inhibition curve was very shallow. See Table II and its discussion.

Routine inhibition titrations were performed by the constant virus-varying inhibitor technic,[8] employing 3 to 4 hemagglutinating doses (HD) of purified swine influenza virus,[3] which had been heated for 30 minutes at 53°C. In these routine tests virus was first incubated with inhibitor for 30 minutes, then red cells were added, and readings were made after an additional hour at room temperature. The inhibition titer of a fluid is expressed as the reciprocal of the final dilution of the fluid in the reaction mixture (composed of 0.5 ml inhibitor dilution, 0.5 ml virus suspension, and 1.0 ml 2% chicken red blood cells) giving the standard two-plus (++) endpoint of hemagglutination. Buffered saline, consisting of 0.81% sodium chloride and 0.005 M phosphate at pH 7.3, was the diluent throughout. Details of other types of inhibition tests are given with the description of the experiments.

*Experimental. Survey.* Samples of RSM, prepared from the individual milks of eight cows, were tested for inhibitory activity against heated swine influenza virus, with the results shown in Table I. Samples of a commercial pool of skim milk before and after pasteurization were included.† As seen in

TABLE II. Effect of Commercial Pasteurization and of Heating at 100°C on the Inhibitory Activity of Milk Against Hemagglutination by Heated Swine Influenza Virus.

| Milk sample | Reciprocal of final dilution of sample | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 40 | 80 | 160 | 320 | 640 | 1,280 | 2,560 | 5,120 | 10,240 | 20,480 |
| Pool 1, raw whole milk | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 3— | 3 | 4— |
| 1, after skimming | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 3— | 3 | 4— |
| 1, skimmed and pasteurized* | 1 | 2— | 2—/2 | 2 | 2/3— | 3— | 3 | 3/4— | 3/4— | 4— |
| 2, raw whole milk | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 3— | 3 | 4— |
| 2, pasteurized† | 0 | 0 | 0 | 0 | 1— | 1 | 2—/2 | 3— | 3 | 4— |
| RSM,‡ 2 min. at 100°C | 1 | 2— | 2 | 2/3— | 3— | 3— | 3—/3 | 3 | 3/4— | 4— |
| ,, 5 ,, ,, | 1 | 2— | 2 | 2/3— | 2/3— | 3— | 3 | 3 | 3/4— | 4— |
| ,, 10 ,, ,, | 1 | 2—/2 | 2 | 2/3— | 3— | 3— | 3 | 3/4— | 3/4— | 4— |
| ,, 15 ,, ,, | 1 | 2—/2 | 2 | 2/3— | 3— | 3— | 3—/3 | 3 | 3/4— | 4— |
| ,, 30 ,, ,, | 2— | 2—/2 | 2 | 2/3— | 3— | 3 | 3 | 3 | 3/4— | 4— |
| ,, 60 ,, ,, | 2—/2 | 2 | 2 | 2/3— | 3— | 3—/3 | 3 | 3 | 3/4— | 4— |

* 30 min. at about 71°C (160°F) by a vat process.
† 20 sec. at about 72°C (162°F) by a continuous flow process.
‡ Raw skimmed milk obtained from Pool 1.
The symbols ± to ++++, usually employed to denote degree of hemagglutination, are replaced for convenience by the symbols 1— to 4. The concentrations of red blood cells in the standard suspensions which are assigned these designations have been shown in a previous publication (Fig. 1 of 10).

[8] Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, **179**, 1275.

† The commercial samples studied in the present investigation were obtained from Durham Dairy Products, Inc., through the courtesy of Mr. V. J. Ashbaugh, Mgr.

Table I, the individual titers are remarkably uniform and have values 1 to 2.5 times as great as those of the commercial pool. Titers similar to these were obtained on all other occasions when individual milks or pools were tested. Accordingly, one may conclude that inhibitory activity is a characteristic property of cow's milk.

*Heat stability of the inhibitor.* Samples of undiluted commercial RSM, prepared by centrifugation, were heated in a boiling-water bath for varying periods, cooled, and titrated for residual inhibitory activity against heated swine influenza virus. Raw and pasteurized samples of whole and skim milk were also tested. The results, presented in Table II, show that the inhibitory activity of RSM is similarly reduced by commercial pasteurization (30 minutes at 71°C) and by heating at 100°C for 2 minutes. Heating at 100°C for periods greater than 2 minutes produces very little further change. Pasteurization of raw *whole milk,* carried out by a different process (20 seconds at 72°C), has little effect on the inhibitor.

From the results with heated RSM, it would appear that the inhibitory activity is associated either with a single substance which has both heat-labile and heat-stable aspects, or with two substances which differ in heat stability. It is interesting that the gradient of inhibition is much more shallow with heated than with unheated skim milk (Table II). On the assumption that the inhibitory activity of milk is associated with a single substance, this difference in gradient is interpreted to mean that the inhibitor becomes weaker, rather than simply more dilute, during heating.[9] Unpublished experiments with the egg-white inhibitor, which is remarkably thermostable, have shown a similar weakening of the inhibitor only after prolonged heating (1 hour or more) at 100°C.

*Mechanism of inhibition.* To determine whether the inhibitor in milk acted on the virus or on the red cells, an experiment was carried out in which RSM dilutions were incubated for varying periods with heated swine virus or with red cells before the second reagent (red cells or virus) was added. Except for this modification, the general set-up of the tests was like that of the routine inhibition titrations. In all cases readings were made one hour after the second reagent was added. The results, plotted in Fig. 1, show that, when RSM dilutions are first incubated with red cells, the inhibition titer is low and independent of time of incubation. In contrast, when RSM dilutions are first incubated with heated virus, the titer, initially low, rises to a high level as incubation is prolonged. This sort of result has been obtained also in similar experiments with egg-white[3] and saliva.[10] It may be concluded that the inhibitor in milk is effective through its capacity to combine with virus.

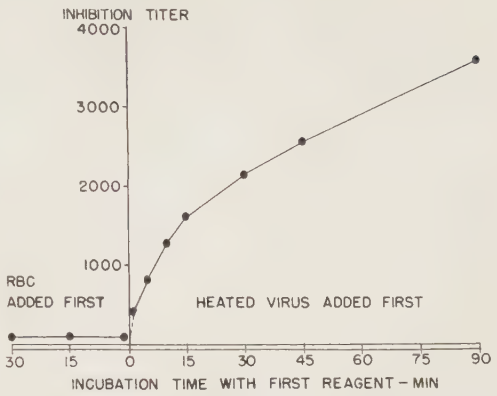


FIG. 1.

Inhibition titer as a function of the order of addition of chicken red blood cells and heated swine influenza virus to dilutions of raw skim milk and the time of incubation at 27°C with the reagent added first.

*Comparison of heated and unheated virus.* Francis[11] showed that the inhibitory effect of normal serum is greater against suitably heated than against unheated virus. This difference in susceptibility of heated and unheated virus to inhibition, explained in terms of a capacity of unheated virus to destroy inhibitor and the loss of such capacity on heating[1,2] has been observed also with milk. Thus, when unheated swine virus was incubated for varying periods with RSM dilutions before the addition of red cells, the conventional inhibition titer rose to about 64 after 5 minutes

9 Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 116.

10 Seltsam, J. H., Lanni, F., and Beard, J. W., *J. Immunol.*, in press.

11 Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

TABLE III. Evidence of Inhibitor Destruction by Unheated Influenza Virus A (PR8 Strain) and Lack of Destruction by Heated Virus.

| Virus PR8 | Incubation with whey,* min. | Total virus nitrogen present, $\gamma$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 | 2 | 1 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |
| Unheated | Control† | 4 | 4 | 4 | 4 | 4 | 3/4— | 3—/3 | 2— | 0/1— | 0 |
| | 1 | 4 | 4 | 3 | 3— | 2— | 0/1— | 0 | 0 | 0 | 0 |
| | 7.5 | 4 | 4 | 3 | 2/3— | 1 | 0/1— | 0 | 0 | 0 | 0 |
| | 15 | 4 | 4 | 3/4— | 2/3— | 1 | 0/1— | 0 | 0 | 0 | 0 |
| | 30 | 4 | 4 | 4— | 3— | 1 | 0/1— | 0 | 0 | 0 | 0 |
| | 60 | 4 | 4 | 4 | 3 | 2— | 0/1— | 0 | 0 | 0 | 0 |
| | 120 | 4 | 4 | 4 | 4 | 3 | 1— | 0 | 0 | 0 | 0 |
| Heated‡ | Control† | 4 | 4 | 4 | 4 | 4 | 3/4— | 2/3— | 2 | 0/1— | 0 |
| | 1 | 4— | 3 | 2/3— | 2— | 1 | 0 | 0 | 0 | 0 | 0 |
| | 7.5 | 4— | 3— | 2—/2 | 1 | 0/1— | 0 | 0 | 0 | 0 | 0 |
| | 15 | 3/4— | 2/3— | 2— | 0/1— | 0 | 0 | 0 | 0 | 0 | 0 |
| | 30 | 3/4— | 2 | 1 | 0/1— | 0 | 0 | 0 | 0 | 0 | 0 |
| | 60 | 3 | 2 | 1— | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 120 | 3 | 2—/2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Final dilution of whey 1:20 in all tubes except controls.
† Saline substituted for whey.
‡ Heated for 30 min. at 53°C at a concentration of 8 $\gamma$ N per ml.

of incubation, and then fell progressively, reaching the value 35 after 90 minutes of incubation. After 30 minutes of incubation, the titer was 48; the corresponding titer against heated virus (Fig. 1) was 2150, about 45 times as great. The decrease in titer with unheated virus after 5 minutes of incubation is interpreted as evidence of inhibitor destruction by virus.

*Inhibitor destruction by virus.* Further evidence of inhibitor destruction by virus is presented in Table III, which shows the results of an experiment carried out with whey and influenza virus A (PR8 strain), purified as previously described.[12] The whey was prepared by bringing RSM to pH 4.6 with N acetic acid, filtering off the precipitated casein, and neutralizing the filtrate to pH 7.0 with NaOH. Progressive dilutions of unheated virus were incubated in constant volume with a constant amount of whey for varying periods at room temperature before red cells were added. The results (Table III) show a slight initial increase in inhibition followed by a progressive decrease, manifested in the partial emergence of the hemagglutinating activity of the virus. Almost identical results were obtained with a partially purified fraction, prepared by half-saturating the whey with $(NH_4)_2SO_4$ (see below); and similar results were obtained with RSM and unheated swine influenza virus. The interesting inhibition optimum seen in Table III has been observed also with saliva[10] and has been explained as a consequence of two oppositely manifested reactions.

Table III shows also the results of a similar experiment carried out with whey and heated PR8 virus. With this virus the inhibition increases progressively as incubation with whey is prolonged, the results emphasizing the difference in behavior of heated and unheated virus noted above.

*Partial purification of the inhibitor.* Preliminary experiments directed at fractionation have shown that the milk inhibitor occurs in the whey and can be recovered almost quantitatively in the precipitate which forms

[12] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

TABLE IV.
Properties of Fractions Obtained from Raw Skim Milk (RSM).

| Fraction (See text) | Inhibition titer* | Nitrogen,* γ per ml | Purification factor† | Activity partition, % |
|---|---|---|---|---|
| RSM | 5,000 | 5,280 | 1.00 | 100 |
| Casein | 410 | 3,830 | 0.11 | 8.2 |
| Whey | 4,300 | 1,130 | 4.0 | 86 |
| P50 | 4,200 | 213 | 21 | 84 |
| P60 | 300 | 55 | 5.8 | 6.0 |
| P100 | 24 | 359 | 0.07 | 0.5 |
| S100 | 11 | 159 | 0.07 | 0.2 |

* Calculated to the original volume of RSM.
† Calculated from the titer-nitrogen ratio with reference to the titer-nitrogen ratio of RSM.

when neutral whey is half-saturated with $(NH_4)_2SO_4$. Less than 1% of the inhibitor is precipitated from neutral whey at 0.33 saturation and less than 10% at 0.4 saturation.‡

An example of the results of fractionation is given in Table IV. A volume of 10 ml N acetic acid was added slowly with vigorous stirring to 100 ml pooled RSM, the pH falling from 6.6 to 4.6. The precipitated casein was obtained by centrifugation, washed with 100 ml 1% NaCl, triturated with 2 ml 10% NaOH, taken up in 100 ml 1% NaCl, and neutralized with N acetic acid. The supernatant whey was neutralized with 2.5 N NaOH and refrigerated for 2 days, during which a slight precipitate formed. This precipitate, which was found to contain about 1% of the initial activity and of the initial nitrogen, was centrifuged off and discarded. The supernatant was brought successively to 0.5, 0.6, and 1.0 saturation with solid $(NH_4)_2SO_4$, added slowly with vigorous stirring. The precipitates, designated P50, P60, and P100, respectively, were taken up in $H_2O$ and dialyzed exhaustively through Visking casing against repeated changes of buffered saline in the cold. The final supernatant at 1.0 saturation, designated S100, was dialyzed against running tap water overnight and then exhaustively against buffered saline in the cold. All of the fractions were analyzed for inhibitor by the routine method and for nitrogen by direct nesslerization.[14] The results show that the inhibitor can be purified about 20 times, with excellent recovery, by this method. These experiments are being continued.

*Discussion.* The non-dialyzability of the inhibitor and its precipitability by salt suggest that the inhibitor is itself a high molecular weight substance, presumably a protein, or is associated with such a substance.

In its reactions with virus, the milk inhibitor displays the characteristic features of other recently discovered inhibitors, such as those occurring in normal serum, normal allantoic fluid, and egg-white. Thus the inhibitor is more effective against heated than against unheated virus and appears to be susceptible to inactivation by the latter. For this reason, and since all samples of raw milk which have been tested possess roughly the same inhibitory activity, it may be concluded that the inhibitor is a normal component of milk rather than a specific antibody produced in response to casual antigenic stimulation. Experiments now in progress in this laboratory indicate that both the milk and the egg-white inhibitors can be distinguished readily in inhibition experiments from the specific antibody which occurs in convalescent anti-influenza swine serum. For example, antibody is equally effective against heated and unheated viruses, while the other inhibitors are considerably more effective against heated virus. Moreover, the gradient of inhibition in the end-point region is much steeper with antibody than with milk or egg-white.

In view of the reported relation between other naturally occurring inhibitors and mucoproteins,[1,2,4,6,8] it is interesting to note that a mucoprotein, designated *lactomucin,* has

‡ A solution containing 7.857 equivalents of $(NH_4)_2SO_4$ per liter is regarded as a saturated solution.[13]

[14] Lanni, F., Dillon, M. L., and Beard, J. W., submitted for publication.

been isolated from milk[13] and that the reported solubility properties of this substance are roughly those of the inhibitor. The relation between the milk inhibitor and lactomucin is being investigated.

*Summary.* Milk is capable of inhibiting hemagglutination by influenza viruses, the inhibitory effect being greater against heated than against unheated viruses. The inhibitor, which is non-dialyzable and moderately heat stable, occurs in the whey and can be salted out by half-saturation with ammonium sulfate. Evidence is presented that the inhibitor is a characteristic component of milk rather than a specific antibody.

[13] Sorensen, M., and Sorensen, S. P. L., *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.*, 1938-41, **23**, 55.

Received Sept. 13, 1949. P.S.E.B.M., 1949, **72**.

## Effect of Dicumarol on Concentration of the Labile Factor.* (17390)

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The observation that the loss of prothrombin activity in stored human plasma could be restored by adding to it rabbit or dog plasma which was markedly depleted of prothrombin by means of dicumarol, led to the discovery of the labile factor and to the assumption that this agent is not affected by dicumarol.[1] The development of a quantitative procedure for estimating the labile factor in blood,[2] now makes it possible to test the validity of this assumption.

*Experimental.* Rabbits and dogs were given dicumarol orally (5 mg per kilo body weight daily) until the prothrombin time became markedly prolonged. The prothrombin activity was determined by the original one-stage procedure.[3] For the estimation of the concentration of the labile factor, the method of Quick and Stefanini[2] was employed. The method consists essentially in determining the prothrombin time of stored plasma after the addition of a measured amount of the plasma to be tested which has been deprothrombinized by adsorption with tricalcium phosphate. The reduction of the prothrombin time is a measure of the concentration of the labile factor.

*Results.* From the data in Table I it can be seen that the concentration of the labile factor in normal rabbit plasma is remarkably constant. This is also true of dog plasma, but the concentration is only one-fifth that of rabbit plasma.[2] When dicumarol is administered the prothrombin activity as measured by prothrombin time decreases progressively and after one week may reach a level below one per cent of normal (Table II). In spite of this drastic decrease, the concentration of the labile factor shows no greater fluctuation than may be attributed to the inherent variations of the method. It can be concluded that dicumarol does not influence the labile factor in rabbits and dogs. Preliminary studies indicate that this is also true in humans.

Owren,[4] Fantl and Nance[5] and Seegers and Ware[6] agree at least tentatively that their respective agents, factor V, accelerator factor and Ac-globulin are the same as the labile factor. The finding of Fahey, Olwin and Ware[7] that dicumarol causes at least a moderate decrease in Ac-globulin in dogs is not in agreement with the present results. It is likely that the difference in findings can

* This investigation was supported by a grant from the Division of Research Grants, National Institute of Health.

[1] Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

[2] Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1948, **33**, 819.

[3] Quick, A. J., *J.A.M.A.*, 1938, **110**, 1658.

[4] Owren, P. A., *Biochem. J.*, 1948, **43**, 136.

[5] Fantl, P., and Nance, M. H., *Med. J. Australia*, 1948, **1**, 98.

[6] Seegers, W. H., and Ware, A. G., *Am. J. Clin. Path.*, 1949, **19**, 441.

[7] Fahey, J. L., Olwin, J. N., and Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 491.

TABLE I.
Concentration of the Labile Factor in Normal Rabbit Plasma.*

| Stored human plasma, cc | | .099 | .098 |
|---|---|---|---|
| Rabbit deprothrombinized plasma, cc | | .001 | .002 |
| | | Prothrombin time (in sec.) | |
| Rabbit | 1 | 14 | 12 |
| " | 2 | 14 | 12 |
| " | 3 | 13½ | 12 |
| " | 4 | 14 | 11 |
| " | 5 | 14½ | 12 |
| " | 6 | 13½ | 12 |
| " | 7 | 14½ | 12½ |
| " | 8 | 13 | 11 |
| " | 9 | 14 | 12 |
| " | 10 | 14 | 12 |

* The stored human plasma had a prothrombin time of 45 sec. The rabbit plasma was treated with tricalcium phosphate gel (0.012 M) to remove prothrombin.

TABLE II.
Effect of Dicumarol Treatment on Concentration of the Labile Factor in Rabbits and Dogs.

| Animal | Day of treatment | Prothrombin time of whole plasma (sec) | Prothrombin time (sec) of mixture: Stored human plasma,† cc / Deprothrombinized plasma of animal treated,‡ cc | .099 / .001 | .098 / .002 |
|---|---|---|---|---|---|
| Rabbit 1* | 0 | 7 | | 13 | 12 |
| | 1 | 11 | | 13 | 11½ |
| | 2 | 8½ | | 13½ | 11 |
| | 3 | 8½ | | 13 | 11 |
| | 4 | 10 | | 12½ | 10½ |
| | 5 | 12 | | 12½ | 11 |
| Rabbit 2* | 0 | 6½ | | 13½ | 12 |
| | 1 | 13 | | 14 | 11½ |
| | 2 | 20 | | 13 | 11 |
| | 3 | 27 | | 13 | 11 |
| | 4 | 53 | | 13 | 11 |
| | 5 | 83 | | 13 | 11 |
| Dog 1 | 0 | 6 | | 20 | 16 |
| | 1 | 11 | | 22 | 15 |
| | 2 | 17 | | 19 | 14 |
| | 3 | 25 | | 18 | 15 |
| | 4 | 39 | | 19 | 15½ |
| Dog 2 | 0 | 6 | | 18 | 14 |
| | 1 | 12 | | 22 | 16½ |
| | 2 | 17 | | 20 | 14½ |
| | 3 | 27 | | 22 | 16 |
| | 4 | 40 | | 20½ | 16 |

* The data were selected to show that the sensitivity to dicumarol bears no relation to variations of the labile factor.

† The stored human plasma had a prothrombin time of 45 sec.

‡ Rabbit and dog plasma were treated with tricalcium phosphate gel (0.012 M) prior to use, to remove prothrombin.

be accounted for by the methods for determining the labile factor (Ac-globulin). With the method of Ware and Seegers[8] the variation of the labile factor in rabbit plasma was found to be from 92 to 310 units and in dogs from 158 to 203 units.[9] In contrast, the method of Quick and Stefanini showed that the labile factor in rabbit plasma was relatively con-

8 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

9 Murphy, R. C., and Seegers, W. H., *Am. J. Physiol.*, 1948, **154**, 134.

stant, and was 5 times higher than in dog plasma.

These differences in results are probably traceable, at least in part, to technical factors of the methods. It appears that the procedure employed in this study is more sensitive and perhaps subject to less error. Nevertheless, when the two methods are applied to normal plasmas they yield results which roughly parallel each other, and both show a low concentration of labile factor in human blood. More important but far more difficult to harmonize and discuss critically are the differences in views concerning the action of the labile factor. Owren, Fantl and Seegers look upon this factor as an accelerator or catalyst, while the writers conclude that it reacts stoechiometrically since a manifold increase in its concentration does not alter the prothrombin time nor the speed of prothrombin conversion.[10] Recently findings have been made which clearly suggest that prothrombin in human blood exists partly in an active form and partly in a precursor state.[11] This discovery introduces a new element in the clotting mechanism requiring evaluation. It is likely that the concept that prothrombin *per se* can change its convertibility or that catalysts can alter it, will require reinterpretation. Since the one-stage method measures active prothrombin, and the two-stage probably total prothrombin, findings such as those reported by Owen and Bollman[12] that the two methods when applied to dicumarolized blood showed marked difference in prothrombin level can be explained without postulating a factor which changes the rate of conversion of prothrombin to thrombin.

*Conclusions.* The labile factor of the prothrombin complex is relatively constant in normal rabbit and dog blood. It is not reduced by dicumarol even after the prothrombin activity falls to a very low level.

[10] Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1949, **34**, 973.

[11] Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1949, **34**, 1203.

[12] Owen, C. A., and Bollman, J. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 231.

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## Intracellular Bile Canaliculi in the Rabbit Liver. (17391)

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There is no controversy as to the general pattern of the bile canaliculi in the mammalian liver with but one exception. This concerns the existence of intracellular branches.

Gomori's[1] technic for the histochemical demonstration of alkaline phosphatase furnishes a very convenient method for the study of the bile capillaries under normal and abnormal conditions, since the bile canaliculi in the liver of some species show striking staining properties.[2-4] The results obtained with this method in the liver of rabbits under normal conditions and after experimental biliary obstruction, strongly suggest the existence of intracellular branches.

*Material and Method.* In medium sized rabbit under ether anesthesia, a liver biopsy was taken and the cystic and bile ducts were ligated. Twenty-two animals were allowed to survive from 1 to 31 days. In addition, livers from normal animals were available. Sections from tissues fixed in cold acetone were stained with Gomori's method[1] as modified by Kabat

[1] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[2] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[3] Wachstein, M., and Zak, F. G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 73.

[4] Jacoby, F., *J. Phys.*, 1947, **106**, 23P.

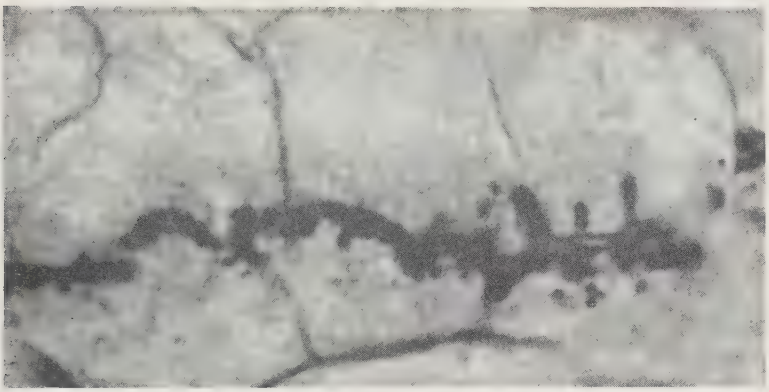

FIG. 1.
Gomori's stain for alkaline phosphatase. Prominent staining of bile capillaries in the normal rabbit liver. Accumulation of granular phosphatase around bile canaliculi. Note intracellular branches. × 1400.

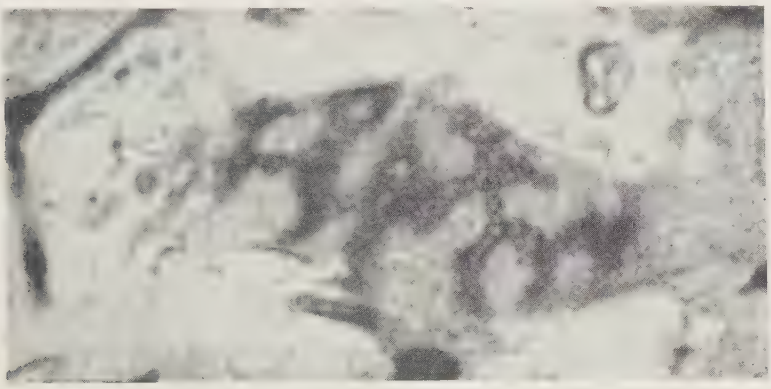

FIG. 2.
Dilatation of bile capillaries 4 days after biliary obstruction. Note intracellular network. × 1400.

and Furth.[5] Sections were incubated for 2 hours.

*Results.* The distribution of alkaline phosphatase activity in the normal liver of the rabbit was quite similar to that previously described in the dog liver.[3] Surrounding the distinctly outlined bile capillaries, granular deposits of alkaline phosphatase were often seen.[6] Fig. 1 shows an intercellularly located bile capillary situated between 2 liver cell cords. From this axial canaliculus, not only intercellular blind ending branches, but also short intracellular ramifications are seen to spring off. These intracellular branches often stain as black lines but occasionally show a distinct lumen. By using the fine adjustment screw under oil immersion, one can be reasonably sure that the short branches lie really within the cell and do not represent portions of communicating intercellular ramifications. Although very short, nob like outpouchings are quite common, larger intracellular branches, as pictured in Fig. 1 are not too frequent in the normal liver. In livers of rabbits which survived the experimental biliary obstruction from 4 to 31 days, however, there occurred not only marked dilation and tortuosity of bile capillaries but intracellular

5 Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

6 Deane, H. W., and Dempsey, E. W., *Anat. Rec.*, 1945, **93**, 401.

branches also became quite numerous. Fig. 2 depicts a network of bile canaliculi located within 2 liver cells. The intracellular branches are seen to possess a real lumen.

*Discussion.* In most modern textbooks of histology, no mention is made of intracellular bile capillaries. Their possible occurrence is conceded in Sharpey-Schafer's[7] and Stöhr's textbooks.[8] Their existence, however, is denied by Maximow and Bloom.[9] The study of finer morphological details with the phosphatase staining technic is considerably helped by the fact that in contrast to the dog liver, the rabbit liver, after biliary obstruction shows no appreciable increase of cytoplasmic phosphatase activity such as often blurs the outline of the dilated capillaries. Clara,[10] using special staining technics, considers the occurrence of intracellular branches in the rabbit liver as likely, but not as definitely, proved. He pointed out that an intracellular location may seemingly be present, while in reality it is due to a location of bile capillaries on the surface of the liver cells in question. While the possibility of this cannot be completely excluded, in the case of the normal rabbit liver, it is quite unlikely in that of the liver after biliary obstruction. Here, a network of dilated bile capillaries is frequently observed on the same plane within a single liver cell. Occasionally, it is found at the level of the cell nucleus.

*Summary.* With the aid of the histochemical phosphatase stain, intracellular bile canaliculi can be demonstrated in the normal rabbit liver. These intracellular branches become considerably more pronounced following experimental biliary obstruction.

7 Sharpey-Schafer, E., Essentials of Histology, Descriptive and Practical for use of students. 13th edition by M. Carleton, London, New York, Toronto.

8 Stohr, P., Lehrbuch der Histologie und der Mikroskopischen Anatomie des Menschen., 25th edition by W. V. Mollendorf, Jena 1943, p. 349.

9 Maximow, A. A., and Bloom, W., Textbook of Histology, Philadelphia and London, 1947, p. 43.

10 Clara, M., *Z. F. Mikr. Anat. Forsch*, 1934, **352**, 1.

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## Enzymatic Hydrolysis of Benzoylarginineamide by Normal and Tuberculous Tissue of Rabbits.* (17392)

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Caseation has been described by Rich[1] in these words, "When, as a result of partial autolysis, necrotic cells lose their structure and outlines, and their remains together with intercellular materials tend to become fused into a formless, coagulated and more or less inspissated mass, the process is termed caseation." In rabbits, experimentally infected with virulent tubercle bacilli, caseation is usually observed about the third week of the disease, when according to Lurie,[2] the monocytes, which make up the bulk of the cellular material in the caseous area, have been transformed into epithelioid cells and allergy to tuberculin has developed. This suggests that the process of caseation is associated with some degree of acquired tissue immunity or resistance to infection with tubercle bacilli.

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1 Rich, A. R., The Pathogenesis of Tuberculosis, C. C. Thomas, Publ., Springfield, Ill., 1946, p. 733 ff.

2 Lurie, M. B., *J. Exp. Med.*, 1933, **57**, 181.

Leading investigators in this field, including Long,[3] Lurie, and Rich, have recognized that the mechanism of caseation and softening is still the key problem in tuberculosis for, as is well known, in the centers of caseous areas tubercle bacilli tend to die, whereas in areas of softening they multiply profusely. As Jaffe[4] expressed it, "The rapid liquefaction of the caseated infiltration is very dangerous, since it opens blood vessels and bronchi. Hemorrhage and aspiration result and the infection spreads rapidly to other portions of the lung." The question arises how does necrotic, caseous tissue in tuberculosis differ from similar material seen in abscess, gumma or infarct? The answer is not yet at hand. Opie and Barker[5] investigated this problem, employing hypertrophied tuberculous lymphatic glands, removed from experimentally infected dogs, as source of enzyme and 50% blood serum, previously heated to 75°C as substrate. They concluded, "When during the third week after inoculation of tubercle bacilli, the figures indicate a maximum degree of digestion, caseation is beginning. . . . After onset of caseation there is gradual and probable complete disappearance of enzymes." Rich[1] summarizes the situation as follows, "At present it remains a problem whether the failure of autolysis to occur in caseous areas is due to an inadequate content of proteolytic enzymes in the monocytes or to the presence of substances that inhibit or destroy the enzymes at the site."

We have studied the behavior of Cathepsin II during various stages of caseation since this endocellular proteolytic enzyme (BA-amidase)[6] is probably concerned in tissue breakdown and in protein synthesis. If this or similar enzymes should play a significant role in the mechanism of caseation and/or softening, there is hope that their action may be influenced by known activators or inhibitors.

*Experimental procedures.* In order to produce caseous tissue, albino rabbits were infected with tubercle bacilli as follows: Animals of Group 1 were injected intratracheally, without previous immunization, with a dose of 0.001 mg of a virulent Ravenel culture of tubercle bacilli. Those of Group 2 were first immunized by means of 2 successive weekly intravenous injections of 1 mg of a lowly virulent R1 strain of *M. tuberculosis* and then 2 weeks later injected intratracheally in the same manner as the animals in Group 1. They were x-rayed at frequent intervals in order to follow the progress of the infection. Five to 8 animals of each group were killed at varying intervals of time—from 5 to 24 weeks after intratracheal infection, by exsanguination from the carotid artery. The organs (lungs, kidneys, liver, and spleen) were removed, weighed, and small sections taken for histologic study. The remainder of the tissues were frozen intact in a dry ice chamber and aliquots (15-20 g) were chipped in the frozen state and placed, together with 10 times their weight of ice water containing chipped ice, into a Waring blendor and homogenized. Under these conditions the blendor remained ice-cold during the one or 2 minutes of homogenation. Aliquots of the homogenates were used for semi-micro-Kjeldahl determinations and enzyme studies. Enzymatic hydrolysis of BAA (benzoylarginineamide) was estimated by the method of Greenstein and Leuthardt,[7] which determines the amount of ammonia N liberated. The reaction constant was calculated from the equation $K = \frac{1}{t} \log \frac{100}{100\text{-}x}$ where x is percentage hydrolysis and t is time in hours. The activity was expressed in units per mg of nitrogen. Under the conditions of these experiments the first order reaction constant for the liberation of ammonia from BAA was proportional to the amount of homogenate used and hence to the enzyme (BA-amidase) concentration.[8]

In carrying out the tests 1.0 ml of organ homogenate was added to 1.0 ml solution of

[3] Long, E. R., *Arch. Path.*, 1939, **28**, 719.

[4] Jaffe, R. H., *Arch. Path.*, 1934, **18**, 712.

[5] Opie, E. L., and Barker, B. I., *J. Exp. Med.*, 1908, **10**, 645.

[6] Bergmann, M., A Classification of Proteolytic Enzymes, in "Advances in Enzymology," edited by F. F. Nord and C. H. Werkman, Interscience Publ., Inc., New York, 1941, **1**, 63-99, and 1942, **2**, 49-68.

[7] Greenstein, J. E., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, 1945-46, **6**, 203.

TABLE I.
Comparison of Benzoylarginine Amidase Activity of Organs of Rabbits Killed at Various Time Intervals After Primary or Reinfection with Virulent Tubercle Bacilli.

| Group | When killed | No. of rabbits | Liver K/g N Avg. | S.E. | Sign | Spleen K/g N Avg | S.E. | Sign | Kidneys K/g N Avg | S.E. | Sign | Lungs K/g N Avg | S.E. | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Normal controls | 14 | 28 | 2.1 | | 24 | 2.7 | | 24 | 1.9 | | 8.6 | 0.8 | |
| | | | | | | After primary infection | | | | | | | | |
| B | 5 wk | 5 | 20 | 1.1 | + | 21 | 3.1 | — | 20 | 1.7 | ++ | 9.8 | 1.4 | — |
| C | 16 wk | 5 | 21 | 0.9 | ± | 31 | 6.9 | — | 25 | 1.4 | — | 11.7 | 4.1 | — |
| | | | | | | After reinfection | | | | | | | | |
| D | 5 wk | 5 | 26 | 1.9 | — | 31 | 2.4 | — | 30 | 4.1 | — | 17.2 | 2.2 | ++ |
| E | 16-20 wk | 5 | 17 | 2.1 | ++ | 27 | 4.2 | — | 38 | 6.0 | ++ | 21.0 | 2.9 | ++ |

S.E. = Standard error (±). Sign—indicates the significance of the experimental averages when compared with the controls. A minus (—) means that the Fischer "t" test gave a "P" value of 0.05 indicating probable significance. A plus (+) denotes a "P" value greater than 0.02 and less than 0.05, indicating statistical significance. Two plus (++) indicates values less than 0.02 or a high degree of significance.

0.1 molar citrate buffer (pH 5.1) containing 0.02 mmols of BAA and 0.01 mmols of cysteine hydrochloride. Digestion was carried out in large test tubes in a 39° bath. After 2 and 4 hours, the tubes were removed, 1 ml of saturated potassium carbonate and 3 ml of water were added and the whole aerated as described by Greenstein and Leuthardt. The ammonia liberated was absorbed in a 2% boric acid solution containing indicator (methyl red-bromcresol green) and titrated with N/14 HCl. A correction blank for zero time was set up in tubes which were treated immediately with saturated $K_2CO_3$.

*Pathologic findings.* The rabbits were killed by exsanguination, at various time intervals after infection, as indicated in Tables I-III. At autopsy the kidneys and lungs showed varying degrees of caseation. Microscopically, these areas were surrounded by inflammatory cells or granulation tissue. There was no caseation in the livers or spleens. In animals which were killed 5 and 16 weeks after primary infection, the lungs were markedly enlarged and weighed from 25 to 135 g as contrasted with an average normal of 10 g. Two protocols are given to illustrate the pathologic findings.

I. *Pathologic findings after primary infection.* Rabbit 8, killed 16 weeks after infection with a virulent Ravenel culture, showed partial consolidation of the lower lobe of the left lung. There were numerous scattered caseous tubercles from 2 to 5 mm in diameter on the surfaces and also within the parenchyma of the lung. The cortices of the kidneys were studded with numerous, firm gray, elevated tubercles, each 2 to 4 mm in diameter. The spleen and liver showed no gross changes.

Microscopically, there was extensive caseation in both lungs and kidneys. The latter also showed extensive necrosis. There was evidence of hemorrhage and some tuberculous involvement in the spleen.

II. *Pathologic findings after reinfection.* Rabbit 66 was reinfected June 1, 1948 after having received 2 intravenous immunizing doses of an R1 culture of *M. tuberculosis* and killed 3 months after intratracheal infection. X-rays, made 6/9/48 and 8/18/48, showed in the fourth interspace of the right lung several areas of density, peripheral to the heart shadow, suggesting consolidation. The remainder of the right lung presented no abnormalities. In the left lung, the bronchial shadows at the hilum of the lung were accentuated. In the fourth interspace there were several spots suggestive of foci of consolidation.

*Gross anatomic findings.* The lungs were well aerated. Several small tubercles were seen on the pleural surfaces. The surface of each kidney showed a single large, prominent tubercle. The spleen was enlarged and showed several tubercles on the surface. The liver was not involved.

*Histological findings.* In sections of the lungs small miliary tubercles were seen whose centers stained well. At the periphery of one tubercle there was an area which was densely

TABLE II.
Illustrative Analytical Data on Normal and Infected Rabbits.

| Rabbit No. | Body wt | Organ | Organ wt | % N | K × $10^3$/ml. hom. | K/g N |
|---|---|---|---|---|---|---|
| | | | Normal animals | | | |
| 9 | 3896 | Li† | 78.3 | 2.87 | 52.2 | 19.3 |
| | | K | 13.0 | 2.53 | 14.7 | 14.8 |
| | | S | 1.39 | 2.37 | 16.0 | 25.8 |
| | | Lu | 11.0 | 2.02 | 12.8 | 8.1 |
| 13 | 4140 | Li | 96.0 | 2.72 | 57.7 | 20.0 |
| | | K | 18.9 | 2.39 | 22.2 | 15.7 |
| | | S | 1.59 | 2.11 | 12.0 | 17.6 |
| | | Lu | 11.74 | 2.12 | 16.1 | 8.4 |
| 18 | 2645 | Li | 98.0 | 2.23 | 58.6 | 18.0 |
| | | K | 12.8 | 2.53 | 22.4 | 15.8 |
| | | S | 1.04 | 3.41 | 3.1 | 8.1 |
| | | Lu | 8.7 | 2.51 | 14.4 | 6.2 |
| 26 | 2870 | Li | 97.3 | 2.55 | 60.5 | 16.9 |
| | | K | 12.8 | 2.46 | 31.2 | 22.0 |
| | | S | 1.02 | 1.76 | 5.6 | 13.6 |
| | | Lu | 9.36 | 2.22 | 11.2 | 6.5 |
| | | | Infected animals* | | | |
| 1 | 3286 | Li | 95.6 | 2.81 | 64 | 24.4 |
| | | K | 24.3 | 2.34 | 85 | 28.5 |
| | | S | 2.7 | 1.48 | 31 | 39.0 |
| | | Lu | 131.0 | 1.80 | 16.3 | 12.5 |
| 2 | 2990 | Li | 64.0 | 2.61 | 30.8 | 18.5 |
| | | K | 16.9 | 2.60 | 49.8 | 22.8 |
| | | S | 1.4 | 2.60 | 18.2 | 12.4 |
| | | Lu | 110.0 | 2.26 | 16.9 | 10.1 |
| 5 | 2590 | Li | 86.8 | 2.35 | — | — |
| | | K | 25.6 | 2.17 | 45.9 | 21.1 |
| | | S | 2.32 | 2.09 | 57.0 | 26.0 |
| | | Lu | 18.2 | 2.02 | 11.0 | 4.5 |
| 6 | 2665 | Li | 73.0 | 2.76 | 45.1 | 21.6 |
| | | K | 15.3 | 2.25 | 45.9 | 27.0 |
| | | S | 2.0 | 2.14 | 20.3 | 15.7 |
| | | Lu | 75.3 | 2.12 | 3.6 | 2.6 |
| 8 | 3296 | Li | 80.9 | 3.06 | 46.6 | 19.7 |
| | | K | 24.6 | 2.17 | 91.1 | 25.5 |
| | | S | 1.56 | 2.58 | 30.8 | 19.0 |
| | | Lu | 26.3 | 1.71 | 43.0 | 25.8 |

* Rabbits killed 16 wk after primary infection with virulent tubercle bacilli.
† Li = Liver; K = Kidney; S = Spleen; Lu = Lung; hom. = Homogenate.

packed with lymphocytes and young fibroblasts. The surrounding lung tissue was fairly normal. There were a few RBC but no fluid. No PMN were seen and not all of the endothelial cells were desquamated. The liver showed no tubercles. There was some congestion and cloudy swelling, also periductal proliferation with monocytes and young fibroblasts. The hepatic lobules were fairly well demarcated as a result of the interstitial reaction. In sections of the kidneys there were many areas of caseation necrosis surrounded by large giant cells. There were also closely-packed collections of cells (monocytes, lymphocytes, and young fibroblasts), the centers of which showed softening. Congestion, cloudy swelling and beginning desquamation of the tubular epithelium were evident in addition to many giant cells. The spleen showed large areas of necrosis with disintegration of the nuclei and absence of cellular outlines. The Malpighian follicles were prominent.

TABLE III.
Total Nitrogen, Protein N, and N.P.N. of Tuberculous and Normal Lungs.

| Rabbit No. | Total N, mg | Total protein, N | N.P.N., mg | N.P.N. as % of total N |
|---|---|---|---|---|
| | | Tuberculous rabbits | | |
| 1 | 2360 | 1950 | 410 | 17.3 |
| 2 | 2500 | 2190 | 310 | 12.4 |
| 6 | 1480 | 1270 | 44 | 14.1 |
| 8 | 446 | 381 | 65 | 14.5 |
| | | Normal rabbits | | |
| 9 | 222 | 202 | 20 | 9.0 |
| 11 | 265 | 251 | 14 | 5.3 |
| 13 | 248 | 222 | 26 | 8.9 |
| 18 | 221 | 206 | 15 | 6.8 |

16 weeks following primary infection the animals showed diffuse caseous consolidation involving both lungs almost completely. These data show that most of the increase in N is due to protein and not to N.P.N.

*Results and discussion.* The biochemical data which are summarized in Tables I-III indicate that homogenates of lungs and kidneys of tuberculous rabbits which are diffusely infiltrated with caseous material are enzymatically very active. While there are individual variations, there is, nevertheless, a statistically significant difference in the rates at which these homogenates hydrolyze BAA which depends upon whether we are dealing with tissues from animals with primary infection or with reinfection. In the former, the rate of hydrolysis of lung tissue was not significantly altered as compared with the normal. Likewise, the kidneys showed either normal rates or moderately decreased activities. After reinfection, however, both lung and kidney homogenates revealed marked increases in hydrolytic activity. These data are strikingly similar to the immunologic results of Lurie,[2] who showed that the lungs and kidneys of normal rabbits possess little innate power to destroy virulent tubercle bacilli after primary infection. But as the result of immunization and reinfection there is more complete destruction of these microorganisms. This increase in bactericidal action is associated with an acceleration of the localized inflammatory process and a heightened physiologic activity of the tissue phagocytes. The liver and spleen, having a high innate capacity to destroy virulent tubercle bacilli, do not call forth any appreciable inflammatory response. The observation that liver homogenates have decreased enzymatic activity after primary as well as reinfection may perhaps be explained by the fact that a loss of liver Cathepsin II may accompany the loss of labile liver protein during the state of malnutrition late in the tuberculous infection.[8] It is also of interest to note that the loss of enzyme activity from the liver takes place earlier following primary infection than following reinfection.

While the data clearly indicate that homogenates of certain organs which contain large amounts of tuberculous caseous material are not enzymatically inert but may show rates of hydrolysis of BAA which are far greater than the corresponding normal values, we have no precise information as to the origin of this added cathepsin. There are 3 possibilities: (a) the inflammatory exudate (or "granulation tissue") which surrounds the necrotic or caseous material is rich in BA-amidase, (b) the caseous material itself, being derived from the breakdown of inflammatory cells is very active enzymatically or (c) the normal, adjacent tissue has acquired an increased rate of hydrolysis. That the first of these two possibilities is the most likely to be true is suggested from the following data:

Ten rabbits which were killed 5 or 16 weeks after primary intratracheal infection with virulent tubercle bacilli had lungs weighing 25 to 125 g, as compared with an average normal value of 10 g. Microscopically, there was caseous and cellular infiltration and chemically there was proof of an accumulation of

[8] Schultz, J., *J. Biol. Chem.*, 1949, **178**, 451.

protein nitrogen (Table III). In spite of this increase in organ weight and the possibility of dilution of enzyme, the rates of hydrolysis of BAA were not significantly different from the normal. Analyses made of bits of caseous material removed and freed from adjacent lung tissue showed them to be enzymatically very active.

The present report confirms the work previously published by Weiss and Halliday[9] that livers and spleens of normal rabbits usually show greater activity of Cathepsin II (BA-amidase) than the lungs. The rate of hydrolysis of lung homogenates is about one-third that of liver. This is of interest since the lungs[2] cannot destroy virulent tubercle bacilli as effectively as do other organs. Considering the size of the liver, it is probably one of the greatest sources of cathepsin in the body.

*Summary and conclusions.* 1. Caseous material was produced in the lungs and kidneys of rabbits by intratracheal infection with a virulent, Ravenel culture of tubercle bacilli. The animals were killed at intervals of from 5 to 20 weeks after infection. The lungs and kidneys, together with the livers and spleens, were removed immediately after exsanguination and death of the animals, preserved at a very low temperature in a dry ice refrigerator, and examined for enzymatic activity.

2. Homogenates of tuberculous lung tissue containing very large amounts of caseous material can hydrolyze benzoylarginineamide (BAA) at pH 5.1 at a more rapid rate than those of homologous normal tissues. The lungs and kidneys of animals which were first immunized with a non-virulent culture before reinfection with a virulent strain of *M. tuberculosis* were found to have greater enzymatic activity than those with a primary infection.

3. These results are analogous to those of Lurie who showed an increased capacity of rabbit tissue to destroy virulent tubercle bacilli under similar circumstances. They are also of interest since we have previously demonstrated[9] a parallelism between bactericidal action and the rate of hydrolysis of BAA in innate organ immunity.

4. It is not clear whether the observed increased rate of enzymatic activity has its origin in the caseous ("necrotic") material itself or in the surrounding inflammatory exudate ("granulomatous tissue"). The following hypothesis is suggested: The lungs and kidneys of rabbits possess little innate resistance to infection with virulent tubercle bacilli. Hence the latter multiply freely and bring about an inflammatory exudate which is the source of the added enzyme. The liver and spleen, on the other hand, are organs of high natural immunity. They do not permit rapid multiplication of tubercle bacilli (Lurie[2]). The intense inflammatory response with enzyme-laden cells is therefore not called forth.

5. The decreased rate in BA-amidase activity observed in liver homogenates after primary and reinfection, may perhaps be due to the loss of labile liver protein which occurs during the malnutrition accompanying the late stages of a virulent tuberculous infection.

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# *In vivo* Acetylation of Natural Amino Acids. (17393)

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Interest in the acetylation of natural amino acids stems from the suggestion that acetylamino acids may be intermediates in protein synthesis.[1] Bloch and Borek[2] demonstrated acetylation of leucine and phenylalanine in liver slices. So far, acetylation of natural amino acids has not been shown *in vivo* probably because in most cases such derivatives are further metabolized rapidly and do not accumulate sufficiently to permit isolation. Knoop[3] found that the unnatural alpha amino acid, phenylamino butyric acid, is readily acetylated in the dog.

Luck and coworkers[4] observed that most of the acetyl-DL-tryptophan administered intravenously to human subjects is excreted unchanged although excretion is negligible when the substance is given orally.[5] Preliminary experiments showed that rats also excrete acetyltryptophan in considerable amounts when it is given intravenously. We have accordingly taken advantage of this behavior to trap any labeled acetyltryptophan formed *in vivo*.

*Experimental methods.* Standard methods were used for the preparation of acetyl derivatives of DL- and L-tryptophan,[6] and acetylglycine.[7] Acetylryptophan was determined colorimetrically by the reaction with p-dimethylaminobenzaldehyde.[4]

*Isolation of acetyltryptophan.* Urine was made alkaline to phenolphthalein and extracted continuously with ether for 24 hours. It was next acidified to congo red and reextracted with ether for another 24 hours. The second ether extract was evaporated to dryness, the residue dissolved in hot water, and decolorized with charcoal. Crystals of acetyltryptophan appeared readily on cooling. Usually, two recrystallizations were enough to give a pure product. Further recrystallizations did not lower the specific radioactivity. The products isolated in experiments I to IV melted at 205-207° (reported 206°) and that in experiments V and VI at 188-189° (reported 190°). The melting points showed no depression on mixing with authentic derivatives. Kjeldahl nitrogen, found 11.28 to 11.46%; theory 11.37%. In order to establish that the radioactivity was due entirely to tryptophan, the isolated acetyltryptophan was hydrolyzed[8] and the convenient formaldehyde derivative of tryptophan prepared.[9] In experiment V 20 mg of acetyl-L-tryptophan was added to the urine as carrier. The specific activity per mole was unchanged (Table I). The derivatives melted sharply at 309-310°. Nitrogen, found 12.88 to 12.99%; theory 12.96%.

*Isolation of acetylglycine.* The method was similar except that ethyl acetate was used instead of ether. Crystallizations have to be carried out with greater care since acetylglycine is more soluble in cold water than acetyltryptophan. The acetylglycine was recrystallized to constant specific activity. It melted at 206°, showed no depression in m.p. on mixing with pure acetylglycine and had a nitrogen content of 11.87% (theory 11.96%).

The samples were deposited on aluminum discs from water or petroleum ether suspension and counted with a thin mica-window Geiger-Muller Counter.

The tryptophan used in the experiments was a racemic mixture labeled with $C^{14}$ on the

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TABLE I. *In vivo* Acetylation of Tryptophan and Glycine.

| Exp. No. | Compounds injected | Cts./min. administered, × 1000 | Acetyl amino acid excreted, mg | Activity cts./min./mg acetyl amino acid | Activity cts./min./mg methylene tryptophan |
|---|---|---|---|---|---|
| I† | Acetyl-DL-tryptophan, DL-tryptophan* | 100 | 75 | 30.7 | 37.6 |
| II† | Acetyl-DL-tryptophan, DL-tryptophan* L-tryptophan (100 mg) | 100 | 58 | 150 | 164 |
| III† | Acetyl-DL-tryptophan, DL-tryptophan* DL-tryptophan (200 mg) | 100 | 40 | 15.8 | 18.1 |
| IV | Acetyl-DL-tryptophan, acetate* | 780 | 49 | 4.4 | 5.5 |
| V | Acetyl-L-tryptophan, acetate* | 780 | 8.7 | 3.7 | 4.1 |
| VI | Acetyl-L-tryptophan, alanine* | 530 | 54 | 5.0 | 5.3 |
| VII† | Acetylglycine, glycine* | 240 | 28‡ | 3.2 | |

* Indicates the radioactive compound.
‡ Final amount recovered pure.
† The data represent the average results from 2 rats. In the rest one rat was used.

beta-carbon and had an activity of 106,000 counts per minute per mg. The alanine was also the racemic compound labeled on the alpha-carbon (600,000 counts per minute per mg). The acetate and glycine were labeled on the carboxyl carbons (210,000 counts per minute per mg and 1,000,000 counts per minute per mg respectively).

In Experiments I to VI, 100 mg of the acetyl-tryptophan (DL- or L- dissolved in 3.8 ml water (pH 7.4) were injected intravenously by the jugular vein immediately followed by intraperitoneal injections of the other compounds mentioned in Table I. In Experiment VII, the rat was injected intravenously with 300 mg of acetylglycine in 3 ml water followed by the labeled glycine solution (0.2 ml) injected intraperitoneally. The urine was collected for the next 8 hours in all cases, the bladder being emptied at autopsy if required.

Acetyl-DL-tryptophan and acetyl-glycine were incubated with labeled tryptophan and glycine respectively for 8 hours in phosphate buffer at pH 7.4 at 37° for 8 hours. The acetylamino acids isolated from the mixture contained no $C^{14}$ showing there was no chemical exchange.

*Results.* The results presented in Table I show that natural amino acids are acetylated *in vivo* in rats. In experiments II and III, the inert L- or DL-tryptophan was added to dilute the specific radioactivity with a view to determining whether the excreted radioactive acetyl derivative was predominantly of the L- or D- configuration. This is of interest since there is no biological mechanism for the deacetylation of D-amino acids.[10] The first four experiments, however, do not definitely decide this question. The subsequent experiments in which acetyl-L-tryptophan was used, leave no doubt that the L-isomer can be acetylated *in vivo*. In the last experiment with acetylglycine, the problem of optical isomerism does not exist.

The radioactivity in the isolated acetyl-L-tryptophan and acetylglycine is rather low. This does not mean that acetylation of amino acids is necessarily a reaction of little im-

[10] Bloch, K., *Physiol. Rev.*, 1947, **27**, 574.

portance. Lack of rapid and complete equilibrium between the newly formed and injected acetylamino acids would also yield low radioactivity in the excreted product. Both acetate and alanine (pyruvate) carbons can be used in this acetylation.

*Summary.* Intravenously injected acetyltryptophan and acetylglycine are excreted in part by rats. Advantage was taken of this behavior to trap any radioactive acetylamino acids that are formed *in vivo*. The results show that natural amino acids can be acetylated *in vivo*.

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## Distribution of Bacitracin in the Body.* (17394)

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Bacitracin is an antibiotic with a wide antibacterial spectrum. It was discovered in the Bacteriological Research Laboratory of the Department of Surgery of Columbia University. The active principle is produced by the Tracey I strain of *B. subtilis*.[1] Bacitracin has been used clinically in the treatment of many infections which had failed to respond to other antibiotic and chemotherapeutic agents.[2] Because of its clinical importance, it seemed to the authors worth while to determine the distribution of the drug in the different tissues and fluids of the experimental animal following intravenous administration.

*First series of experiments.* Eleven normal rabbits weighing from 2 to 4 kilos were selected. Bacitracin in a dosage of 3,000 units per kilo of body weight, dissolved in 3 or 5 cc of physiological saline, was injected slowly into the ear vein of each rabbit. One, 2 or 3 hours after the injection the animals were bled to death under nembutal anesthesia. The whole organ or a portion of the tissue was then removed from the skin, heart, lung, liver, skeletal muscle, brain, bone marrow, pancreas, kidney, intestines and the spleen. Each kind of tissue was ground up in a mortar with an equivalent weight of physiological saline. It was then filtered through cotton. Each filtrate was then assayed for the concentration of bacitracin by the penicylinderplate method against *Micrococcus flavus*.[3] The cerebrospinal fluid, blood, bile and urine were also collected and their bacitracin content similarly determined. The results are presented in Table I.

*Discussion.* Although there are some wide variations, composite figures as expressed in averages give an approximate value for the various tissues. The figures seem to indicate that the antibiotic is widely distributed throughout the body, but its concentration is somewhat variable in different tissues. For example, the chorioid plexus apparently acts as a barrier to the passage of bacitracin. None or only a trace of bacitracin was found in the cerebrospinal fluid or in the brain tissues after intravenous or parenteral injection. This is also true of penicillin and streptomycin.[4,5]

* This study was supported in part by a grant from the Medical Research and Development Board of the Office of the Surgeon General of the Army.

The bacitracin used was supplied by the Commercial Solvents Corporation of Terre Haute, Ind.

1 Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

2 Meleney, F. L., Altemeier, W. A., Longacre, A. B., Pulaski, E. J., and Zintel, H. A., *Ann. Surg.*, 1948, **128**, 714.

3 Hott, D. A., Bennett, R. E., and Stanley, A. R., *Science*, 1947, **106**, 551.

4 Struble, G. G., and Bellows, J. G., *J.A.M.A.*, 1944, **125**, 685.

5 Buggs, C. W., Pilling, M. A., Bronstein, B., Hirshfeld, J. W., Worzniak, L., and Key, L. J., *J. Clin. Invest.*, 1946, **25**, 94.

TABLE I. Concentration of Bacitracin in Body Tissues and Fluids of 11 Rabbits After a Single Large Intravenous Injection of Bacitracin. (3,000 units/kilo body wt).

| Tissue or fluid | Units of bacitracin per g or cc | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sacrificed after 1 hr | | | | Sacrificed after 2 hr | | | | Sacrificed after 3 hr | | | |
| Rabbit No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | |
| Wt in kg | 2.5 | 2.0 | 2.5 | 2.5 | 2.0 | 2.5 | 2.5 | 2.0 | 4.0 | 3.5 | 4.0 | Avg |
| Blood | 3.65 | 18.0 | 2.55 | 10.0 | 8.55 | 1.25 | 1.6 | 6.2 | 10.0 | 4.76 | 5.1 | 2.56 | 3.8 | 3.82 |
| Bile | 1.24 | 1.8 | 1.5 | 1.4 | 1.46 | 2.61 | 0.64 | 1.8 | 2.85 | 1.97 | 0.93 | 1.02 | 0.8 | 0.9 |
| Cerebrospinal fluid | 0.1 | 0.1 | 0 | 0 | Trace | Trace | Trace | 0.1 | Trace | Trace | 0 | Trace | Trace | Trace |
| Urine | 177.0 | 57.5 | 180.0 | | 138.16 | 325.0 | 15.0 | 4.6 | 65.0 | 102.4 | 11.2 | 78.0 | 17.0 | 35.4 |
| Kidney | 3.9 | 3.1 | 1.98 | 5.4 | 3.59 | 2.61 | 1.41 | 4.2 | 12.4 | 5.15 | 6.0 | 5.4 | 2.8 | 4.7 |
| Lung | 1.35 | 2.0 | 0.7 | 1.25 | 1.32 | 0.45 | 0.25 | 1.35 | 5.4 | 1.86 | 0.8 | 0.62 | 1.32 | 0.91 |
| Pancreas | 0.1 | 0.7 | 0.34 | 0.31 | 0.36 | 0.28 | 0.1 | 0.32 | 1.84 | 0.63 | 1.2 | 0.4 | 0.38 | 0.66 |
| Skeletal muscle | 0.53 | 0.7 | 0.1 | 0.23 | 0.39 | 0.1 | 0.1 | 0.29 | 1.53 | 0.5 | 0.42 | 0.31 | 0.1 | 0.27 |
| Heart | 0.36 | 1.35 | 0.3 | 0.75 | 0.69 | 0.3 | 0.1 | 0.43 | 1.08 | 0.47 | 0.42 | 0.29 | 0.34 | 0.35 |
| Spleen | 0.39 | 0.9 | 0.1 | 0.48 | 0.47 | 0.1 | 0.1 | 0.23 | 0.6 | 0.26 | 0.44 | 0.31 | 0.24 | 0.33 |
| Liver | 0.27 | 0.72 | 0.1 | 0.48 | 0.39 | Trace | 0.1 | 0.51 | 0.96 | 0.39 | 0.44 | 0.1 | 0.26 | 0.27 |
| Brain | Trace | 0 | 0 | Trace | Sl. Tr. | Trace | 0 | Trace | 0 | Sl. Tr. | Trace | 0.1 | 0 | Trace |
| Bone marrow | 0.84 | 0.72 | 0.66 | 0.91 | 0.78 | 0.7 | 0.54 | 0.3 | 3.12 | 1.16 | 0.96 | 0.41 | 0.7 | 0.69 |
| Skin | 0.82 | 3.6 | 0.38 | 0.65 | 1.36 | 1.05 | 0.12 | 0.5 | 1.7 | 0.84 | 0.58 | 0.13 | 0.48 | 0.39 |
| Large intestine | 0.42 | 2.85 | Trace | 0.80 | 1.0 | 0.1 | 0.1 | 0.67 | 1.92 | 0.69 | 0.24 | | 0.22 | 0.23 |
| Small intestine | 0.33 | 0.33 | 0.34 | 0.42 | 0.35 | 0.1 | 0.1 | 0.24 | 1.0 | 0.36 | 0.28 | | 0.42 | 0.35 |

However, this barrier is overcome when the meninges are severely inflamed, as in suppurative meningitis, for then a significant level of bacitracin can be recovered in the cerebrospinal fluid.[6]

The high bacitracin content in the kidney is to be expected in view of the fact that this organ is active in the excretion of the drug. The concentration of bacitracin in the urine obviously depends to a considerable degree upon both the total intake and the total output of fluid. The output of urine could not be determined because the animals voided a variable amount during the course of the experiment and the urine was not collected. The concentrations noted in the table were those found in the urine present in the bladder at the time of death. The wide variation in these figures was not unexpected for it is similar to that found in patients following intramuscular injection. Although no albumin or casts were found in these urine specimens and there was no gross evidence of kidney damage at the time of autopsy, it is possible that there may have been some variation in renal blood flow or glomerular or tubular filtration to account for the wide variations in the urine concentrations. This might also explain the wide range in the blood levels.

Of interest and possibly of clinical importance is the relatively high bacitracin content in the lungs, the skin and the bone marrow. The content of the drug in the bone marrow is in contrast to that of penicillin, which according to the report of Struble and Bellows does not reach this tissue.[4] However, some doubt is cast upon this finding with regard to penicillin by the excellent response of acute osteomyelitis to treatment with this drug. This might be explained on the basis of increased permeability of the capillary walls in the early stages of an acute inflammation. The high figure for bile may be partially explained by the fact that a control specimen of bile showed an inhibitory effect against the growth of *Micrococcus flavus*. However the variation in the levels, with the peak average at two hours, suggests that bacitracin is ex-

[6] Teng, P., and Meleney, F. L., accepted for publication in *Surgery*.

TABLE II.
Concentration of Bacitracin in the Chest and Peritoneal Fluids and in the Blood Serum of Rabbits after an Intravenous Injection of the Antibiotic.* (3,000 units/kilo body wt).

| Rabbit No. | Bacitracin levels in units/cc | | |
|---|---|---|---|
| | Chest fluid | Peritoneal fluid | Blood serum |
| 1 | 2.3 | 2.3 | 1.6 |
| 2 | 5.0 | | 7.5 |
| 3 | 4.2 | | 4.5 |
| 4 | | 2.6 | 2.55 |
| 5 | 2.4 | | 2.8 |
| Avg | 3.47 | 2.45 | 3.79 |

* One hour after bacitracin injection and 25 hours after the intraperitoneal and intrathoracic injections of aleuronat-starch.

creted in the bile.

There was a moderate concentration of bacitracin in the wall of both the small and the large intestine. This suggests the possible value of the antibiotic in the treatment of intestinal infections, either bacterial or protozoan, provided that these infections are caused by organisms susceptible to bacitracin.

The liver, spleen, pancreas, heart and skeletal muscles all showed a moderate amount of the drug, which suggests that infections in these organs might be reached by the circulating drug.

*Second series of experiments.* In the second part of this study, 10 cc of aleuronat-starch, which contained 3% starch and 5% aleuronat, were injected into the pleural and into the peritoneal cavities of 5 normal rabbits 24 hours before the intravenous administration of bacitracin in the same dosages described above. These animals were sacrificed one hour after the injection of the antibiotic. The pleural or peritoneal exudate produced by the irritation of the aleuronat-starch was collected for a study of the bacitracin level by the penicylinder-plate method. The results are shown in Table II.

*Discussion.* These figures seem to indicate clearly that bacitracin diffuses into the exudate in the purulent response to a chemical irritant in experimental rabbits and presumably will diffuse into an exudate of infectious origin in man. In one rabbit, the bacitracin levels in the peritoneal and pleural fluid were higher than those in the blood serum. In one the level in the chest fluid was lower, and in the others the titres were approximately the same as in the blood serum. Johnson, Anker and Meleney reported that subcutaneous bacitracin could save experimental mice following the intraperitoneal injection of 10,000-100,000 minimum lethal doses of hemolytic streptococci.[1]

In 3 clinical cases of ascites, from 1 to 6 hours after a single intramuscular injection of 49,000 units of bacitracin, which was approximately 1,000 units per kilo of body weight, Michie found that the concentration of the drug varied from 0.0018 to 0.128 unit per cc of ascitic fluid. In the pleural fluid of one patient he demonstrated a level of 0.5 unit per cc 4 hours after an intramuscular injection of 40,000 units of bacitracin.[7]

The fluid collected from the above patients was of a transudative nature. In the case of exudates, a higher bacitracin content might well be expected. Whenever the permeability of blood vessels is increased by an inflammatory process, it is easier for bacitracin to pass through the capillary bed and enter the inflamed tissues.

During the early commercial production of bacitracin, certain lots showed a disturbing degree of kidney irritation which interfered with systemic administration, but since July of 1948 our experience has shown that a product which meets the Food and Drug Administration's specification for toxicity of an LD50 of 500 units for 20-g mice, produces only a minimal and transient degree of kidney irritation. The lot used in these experiments, namely, No. 480408, met that specification and has been used without difficulty by intramuscular injection in a large number of human infections.

*Summary.* One, 2 or 3 hours after a large single intravenous injection of bacitracin, the concentration of the drug in the different tissues of rabbits was found in decreasing order as follows, subject to some individual variation: urine, kidney, blood, bile, lung, bone marrow, skin, large intestine, pancreas, heart muscle, skeletal muscle, liver, spleen,

[7] Michie, A., work in progress.

small intestine, cerebrospinal fluid and brain.

In the chest and peritoneal exudates produced by aleuronat-starch injection, the titres of bacitracin one hour after an intravenous administration approximated those in the blood.

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## Production of an Inactive Derivative of Purified Prothrombin by Means of Purified Thrombin.* (17395)

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The blood clotting mechanism is of such complexity that the reactions which the several clotting factors can participate in may be followed with certainty only when these factors are isolated and obtained in purified form. With the use of such purified preparations, it has been shown that, depending upon the conditions of the experiment, thrombin may have entirely different effects on prothrombin. When small amounts of thrombin are added to prothrombin dissolved in 25% sodium citrate solution, the rate of activation of the prothrombin to thrombin is increased;[1,2] when thrombin and prothrombin react in physiological saline solution the prothrombin is changed to an inactive form in which it does not react to the addition of calcium, Ac-globulin and thromboplastin.[3,4] These effects are apparently produced consecutively when relatively large amounts of thrombin are used. There first appears a decrease in sensitivity to calcium, Ac-globulin and thromboplastin, then a reappearance of the original sensitivity. These experiments might be interpreted to mean that prothrombin is inactivated only to be changed back to its original active form again. It appears more reasonable, however, that thrombin acts first to form a slightly modified prothrombin which is not reactive to thromboplastin, and that this modified prothrombin is then changed to another active form of prothrombin which yields thrombin. The sequence may be described by the following: Prothrombin (sensitive to calcium + Ac-globulin + thromboplastin) → Prothrombin-derivative I (not sensitive to calcium + Ac-globulin + thromboplastin) → Prothrombin-derivative II (sensitive to calcium + Ac-globulin + thromboplastin) → Thrombin and possibly other reaction products. In this paper we shall show that the first prothrombin-derivative, a protein insensitive to calcium + Ac-globulin + thromboplastin, can be identified by electrophoresis.

*Experimental procedures.* A large homogeneous supply of prothrombin was obtained by pooling several preparations made as previously described.[4-6] Many experiments were performed but all of those described in this paper were performed on this pooled sample of prothrombin.

When dissolved in saline solution it retained at least 95% of its activity for 6 hours at room temperature. On electrophoresis the patterns (Fig. 1) showed that 95% of the total protein was present in one component in the descending boundary, while 87% was present in the ascending boundary. The impurities present in the prothrombin sample were thus shown to be sufficiently reduced to

* Aided by a grant from the United States Public Health Service, National Institute of Health. Parke, Davis and Company supplied large quantities of plasma required for the preparation of purified prothrombin and thrombin.

1 Seegers, W. H., and Ware, A. G., *Fed. Proc.*, 1949, **8**, 249.

2 Seegers, W. H., *Am. Heart J.*, in press.

3 Mertz, E. T., Seegers, W. H., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 657.

4 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **174**, 565.

5 Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

6 Seegers, W. H., *J. Biol. Chem.*, 1940, **136**, 103.

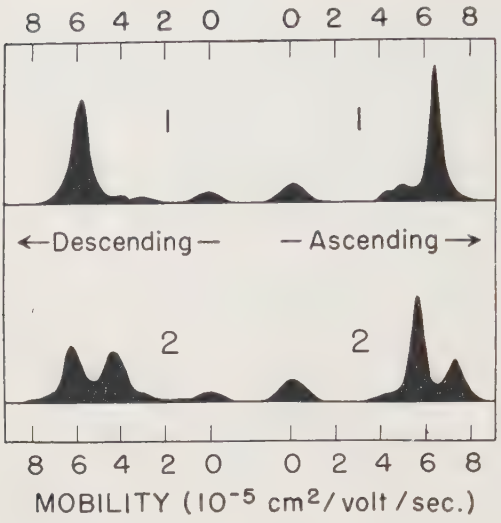


FIG. 1.

The electrophoretic boundary patterns of purified bovine prothrombin and of the same prothrombin after partial inactivation with purified thrombin, in barbital buffer, pH 8.5, ionic strength 0.1, and after 150 min. electrophoresis.

permit a study of the effect of thrombin on the boundary patterns and electrophoretic mobility of prothrombin.

The purified thrombin was prepared by dissolving sufficient purified prothrombin in a 25% solution of sodium citrate to make a 1% solution, adding a small amount of 3-methyl-4, 6, 4′-triaminodiphenyl sulfone and allowing the solution to stand at room temperature for 24 hours. The addition of the sulfone increases the yield of thrombin to a maximum. To separate the formed thrombin from the other substances present it was precipitated by the addition of ammonium sulfate, redissolved in water, and dialyzed free of salt. This method of preparing thrombin will be described in detail and presented for later publication. The important feature of this method is that thrombin is obtained from prothrombin without the use of calcium, Ac-globulin or thromboplastin and is thus free of these substances and other impurities present in even the best Ac-globulin and thromboplastin preparations now available. Thrombin prepared in this way is of higher purity than any previously described, and is of sufficient activity that in the amounts used in our reaction mixtures it could not be detected electrophoretically in the presence of the prothrombin.

Reaction mixtures were prepared from these purified preparations by dissolving in physiological saline sufficient prothrombin to give a concentration of 7,700 units per cc of solution and adding sufficient dry thrombin to bring its concentration to 100 units. The reaction mixture was allowed to stand for 6 hours at room temperature, when samples were removed for measurement of prothrombin activity, by the modified two-stage method[7,8] of analysis and the remainder of the reaction mixture was frozen, dried from the frozen state, dissolved in barbital buffer at pH 8.4 and 0.1 ionic strength and subjected to electrophoresis. The prothrombin activity decreased during the period of incubation to 62% of the original value. Comparison of the electrophoretic patterns of the prothrombin before and after incubation with thrombin (Fig. 1) reveals that the major component of the original sample was reduced in quantity from 87% to 32% in the ascending boundary and from 94% to 53% in the descending boundary. This protein which has disappeared from the major boundary reappeared in a second boundary which represented 64% of the total protein in the ascending boundary and 43% in the descending boundary. The decrease in prothrombin activity as measured by the two-stage method of analysis was in rough agreement with the shift of protein from the main electrophoretic component to the second component. The inactive derivative of prothrombin is characterized by an electrophoretic mobility of 5.60 (ascending) and 4.21 (descending) $\times 10^5$ cm²/volt/sec.

One of the principal difficulties encountered in early work[9] on the purification of prothrombin was the loss of prothrombin activity

[7] Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, **19**, 471.

[8] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

[9] Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 58.

due to the action of small amounts of thrombin, formed spontaneously from the prothrombin. It was not appreciated at the time that the product formed from the prothrombin was the principal impurity of the preparation. The thrombin produced a derivative of prothrombin which was without typical activity. It is likely that the presence of this inactive prothrombin derivative is responsible for the polydisperse appearance of the prothrombin preparations examined in the ultracentrifuge.[10]

*Summary.* When purified prothrombin was allowed to react with a small amount of purified thrombin there was loss in prothrombin activity. Comparison of the electrophoretic patterns of the prothrombin before and after alteration with thrombin showed that protein disappeared from the curve representing prothrombin and appeared as a new component with a lower electrophoretic mobility than that of prothrombin.

[10] Seegers, W. H., and Ware, A. G., *Fed. Proc.*, 1948. **7**, 186.

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## Acetylcholine and Blood Sugar. (17396)

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Intravenous injection of acetylcholine (100-500 $\alpha$/kg, i.v.) produces hyperglycemia (25-60%) in rabbits. High doses of acetylcholine (500-1000 $\alpha$/kg, i.v.) induce convulsions and a marked increase in blood sugar, up to 200%.

The mechanisms of the hyperglycemia provoked by acetylcholine have been investigated in a series of experiments. The observations may be summarized as follows:

1. Atropine (0.25 - 1 mg/kg, i.v.) does not abolish the acetylcholine hyperglycemia

2. The adrenolytic drug SY 28 ($\alpha$-naphthyl-methyl-ethyl-$\beta$-bromo-ethylamine HBr), in doses of 1 to 3 mg/kg, does not abolish the hyperglycemia induced by acetylcholine or epinephrine.

3. The anticholinesterase drug:dimethyl-carbamate of hydroxyphenyl-benzyl-trimethyl-ammonium (Nu-683) (0.1 - 0.3 mg/kg i.v.) sensitizes the animal very markedly to the hyperglycemic actions of acetylcholine.

4. Tetraethylammonium (20 mg/kg i.v.) protects against acetylcholine-hyperglycemia, if doses of acetylcholine which do not induce convulsions are injected.

5. After removal of both adrenal glands and treatment with desoxycorticosterone-acetate, rabbits do not present hyperglycemia after doses of acetylcholine which do not induce convulsions. Higher doses of acetylcholine inducing convulsions still provoke hyperglycemia.

6. Nembutal (Sodium-ethyl(-l-methyl-butyl) barbiturate) anesthesia prevents acetylcholine-hyperglycemia.

7. Control experiments show that no changes in blood sugar occur after injections of the same doses of atropine, tetraethylammonium SY 28 or Nu-683.

8. High doses of Nu-683 (0.5 mg/kg) induce hyperglycemia, which is also prevented by tetraethylammonium.

*Conclusions.* In rabbits, doses of acetylcholine which do not provoke convulsions induce hyperglycemia.

This increase in blood sugar is prevented by the synaptic blocking agent tetraethylammonium, by nembutal and by removal of both adrenal glands.

The acetylcholine-hyperglycemia thus is induced by adrenal synaptic stimulation, which increases epinephrine output.

The acetycholine and epinephrine hyperglycemia are not abolished by an adrenolytic agent (SY 28). The hyperglycemia induced by high doses of an anticholinesterase drug (Nu-683) is also prevented by tetraethylammonium.

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# Goiterogenic Action of Iodide and the Etiology of Goiters in Chicks from Thyroprotein-fed Hens. (17397)

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It has been shown recently that the feeding of synthetic thyroprotein (STP) to hens increases the time required to hatch their eggs and results in the presence of goiters in the chicks hatched from these eggs.[1,2] These chicks are apparently hypothyroid as shown by their lowered oxygen consumption rate.[3] These results are contradictory because STP fed directly to adults and growing stock[4,5] results in an elevated metabolic rate and in thyroid involution. Injection of STP into fertile eggs also results in thyroid involution.[6] In the present communication, a mechanism involved in this paradox is established. It had been postulated that maternal thyroxine deposited in the egg regulates the thyroid function of the embryonic chick. Thus the goiters in chicks from STP-fed dams were believed due to reduced maternal thyroid activity with attending failure of the thyroidal substance in STP to be deposited within the egg. This hypothesis proved untenable, however, when the feeding of desiccated thyroid to hens failed to alter incubation time of their eggs or thyroid size of their chicks.[7]

A new hypothesis based on the relatively high inorganic iodide content of STP was developed following the report by Wolff and Chaikoff that the administration of large amounts of inorganic iodide inhibited thyroxine synthesis in the thyroid gland of the normal rat.[8] In these experiments thyroid inhibition was of short duration and it is not surprising that thyroid enlargement did not occur. However, it might be expected that prolonged inhibition of the thyroid by any agent would result in a compensatory enlargement. As a result of our efforts to test this thesis, it has been demonstrated that inorganic iodide can act as a goiterogenic agent.[9] When large amounts of inorganic iodide were injected into the white of fertile eggs on the 16th day of incubation, the chicks exhibited marked thyroid enlargement at the time of hatch. Moreover, these goiterous chicks exhibited typical hypothyroid symptoms: delayed hatching and delayed closure of the umbilicus.

To establish inorganic iodide as the cause of goiters in chicks from STP-fed dams, it was necessary to demonstrate that the inorganic iodide content of the hen's diet could affect the thyroid size of her chicks. This might be expected since the iodide content of the hen's diet determines to a large extent the iodine content of her eggs.[10] In the commercial manufacture of STP, casein is incubated with elemental iodine and the resulting product contains 1.5-2.0% inorganic iodine as iodide as well as 3.0% crystalline thyroxine.[11] Hence, it seems logical that this inorganic iodide in STP might increase the iodide content of the egg sufficiently to inhibit thyroxine synthesis in the embryonic chick thyroid and produce a compensatory thyroid enlargement.

*Experimental.* To test this hypothesis, groups of 12 hens were fed a conventional diet

[1] Wheeler, R. S., and Hoffmann, E., *Endocrinology*, 1948, **42**, 326.

[2] Wheeler, R. S., and Hoffmann, E., *Endocrinology*, 1948, **43**, 430.

[3] McCartney, M. G., and Shaffner, C., *Poultry Sci.*, 1949, **28**, 223.

[4] Wheeler, R. S., Hoffmann, E., and Graham, C. L., *Poultry Sci.*, 1948, **27**, 102.

[5] Wheeler, R. S., and Hoffmann, E., *Poultry Sci.*, 1948, **27**, 509.

[6] Booker, E. E., and Sturkie, P. D., *Poultry Sci.*, 1949, **28**, 147.

[7] Brooke, J. F., and Wheeler, R. S., unpublished data.

[8] Wolff, J., and Chaikoff, I. L., *Endocrinology*, 1948, **42**, 468.

[9] Wheeler, R. S., and Hoffmann, E., *Endocrinology*, 1949, **45**, 208.

[10] Wilder, O. H. M., Bethke, R. M., and Record, P. R., *J. Nutrition*, 1933, **6**, 407.

[11] Graham, W. R., Jr., personal communication. Cerophyl Laboratories, Kansas City, Missouri.

TABLE I.
Thyroid Weight of Adult Hens Fed Diets Containing Large Amounts of Inorganic Iodide, and Thyroid Weight of Their Progeny on Day of Hatch.

| Diet of hens | | Amt thyroxine added to diet, (mg/lb) | Amt inorganic I, added to diet, (mg/lb) | Mean thyroid wt* of chicks, (mg ± S.E.) | Mean thyroid wt† of hens, (mg ± S.E.) |
|---|---|---|---|---|---|
| Control | | — | — | 3.0 0.06 | 98 16.9 |
| .04% | STP‡ | 6 | 4 | 7.2 0.22 | 22 0.7 |
| .001% | KI | — | 4 | 7.0 0.15 | 149 45.9 |
| .002% | ,, | — | 8 | 8.1 0.12 | 162 16.9 |
| .005% | ,, | — | 16 | 8.5 0.33 | 143 43.9 |
| .009% | ,, | — | 32 | 8.4 0.32 | 352 30.0 |
| .018% | ,, | — | 64 | 12.6 1.18 | 330 63.9 |

* Avg of thyroids from 10 day-old chicks.

† Avg of paired thyroids from 7 hens except in groups fed 32 and 64 mg I per lb of feed, which included 6 and 5 paired thyroids, respectively.

‡ STP, trade name Protamone, furnished by Cerophyl Laboratories, Kansas City, Mo.

supplemented with varying amounts of iodide (as KI), or STP. It is necessary to emphasize the excessive nature of the iodide levels. To meet the recommendation of the National Research Council, practical poultry diets should contain 0.5 mg iodine per pound. The diets employed in the present study contained 8 to 128 times this amount The results are presented in Table I. It will be observed that the feeding of KI induced thyroid enlargement in the adult hens, confirming the goiterogenic action of excessive amounts of inorganic iodide which had been previously demonstrated in the chick embryo. As expected, STP caused thyroid involution in the adults, indicating that the thyroxine intake was sufficient to inhibit thyrotropin secretion.

The offspring of KI-fed hens also showed marked thyroid enlargement. It is of particular interest that hens fed approximately the same amount of iodide as was supplied by STP, produced chicks with thyroid enlargement comparable to that in the offspring of the STP-fed hens. Apparently the thyroid enlargement found in chicks from STP-fed hens is associated with the iodide rather than with the thyroxine in STP. This would explain why the feeding of desiccated thyroid,[7] or STP[1,2] to hens, or the injection of crystalline thyroxine into hens,[3] fails to decrease thyroid size in their chicks. Apparently any thyroxine naturally deposited in the egg is metabolized or inactivated by the time the chick thyroid becomes functional (about the 14th day of incubation) and, therefore, fails to modify thyroid size. It should be noted, however, that if STP[6] or thyroxine[3,6] is injected directly into eggs during the first week of incubation, chick thyroid size at time of hatch is significantly reduced.

The hypothyroid symptoms of offspring of hens fed inorganic iodide are essentially the same as those in chicks from eggs injected with thiourea,[12] or KI[9]; in chicks from dams fed STP;[12] and in squabs from iodine-deficient dams.[13] The delay in hatching caused by feeding iodide to hens may explain the findings of Wilgus, *et al.*[14] that hatchability was unfavorably affected by amounts of iodine in the hen's diet in excess of 23 mg/lb. In the present study no decrease in hatchability was noted if the eggs were left in the incubator an extra day to allow for the delayed-hatching effect of the iodide supplement.

Further evidence of the like effects of feeding KI and STP to the hens on the thyroid glands of newly hatched chicks has been obtained from histological sections. (Fig. 1.) The thyroid glands of chicks whose dams were

12 Grossowicz, N., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 151.

13 Hollander, W. F., and Riddle, O., *Poultry Sci.*, 1946, **25**, 20.

14 Wilgus, H. S., Gassner, F. X., Patton, A. R., and Harshfield, G. S., *Poultry Sci.*, 1948, **27**, 686, (abstract).

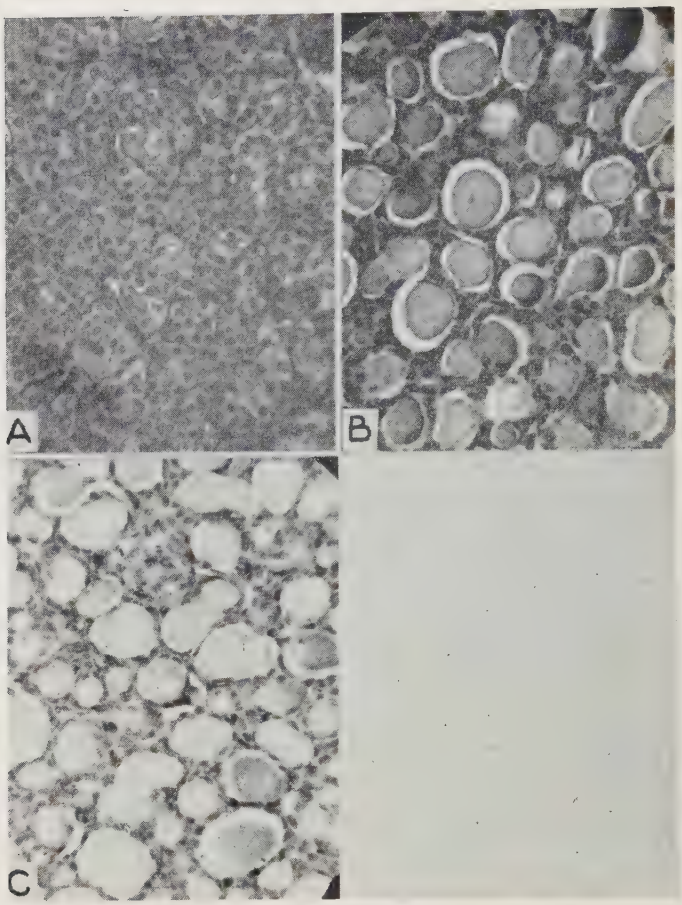


FIG. 1.
Histological sections of the thyroid gland of day-old chicks 225 X. A. Normal chick thyroid. B. Thyroid of chick from STP-fed hen. C. Thyroid of chick from KI-fed hen.

fed either KI or STP are indistinguishable; the many follicles are distended with colloid and the epithelial cells are flattened. In the normal gland very few follicles are seen and the epithelial cells are cuboidal.

Histological sections of the thyroid glands of hens fed KI are of interest because of marked colloid storage. (Fig. 2.) In most cases the follicles were so large that it was not possible to photograph a sufficient area to convey the true picture. It would appear that the generally accepted concept of Marine[15] that colloid goiter is the result of involution of a previously hyperplastic gland, cannot explain the tremendous enlargement observed in the present study after only one month of treatment. In 3 cases where hens received the 2 highest dosages of KI, goiters with colloid nodules and cystic degeneration were observed. A line drawing of one of these glands is presented in Fig. 3. Apparently the follicles become confluent and form large colloid vesicles surrounded by a pseudo encapsulation. There was no evidence of neoplasm. However, the trabeculae forming the boundaries of the vesicles contained some thyroid tissue which appears to be normal and some thyroid tissue showing only slightly increased colloid storage. The weights of these glands were not included in the data because of their extreme deviation from the mean. The largest gland was 50 x 35 mm and the

[15] Marine, D., *Medicine*, 1924, **3**, 453.

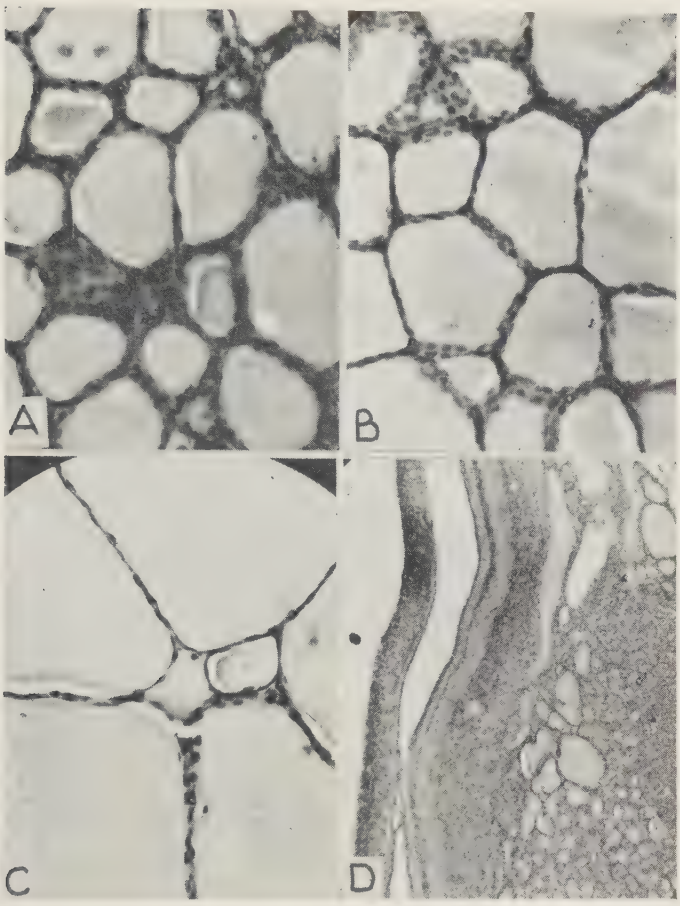


FIG. 2.

Histological sections of the thyroid gland of laying hens. A. Thyroid of normal hen. B. Thyroid of STP-fed hen. C. Thyroid of KI-fed hen. D. Low power field of the "normal" thyroid tissue and pseudo-encapsulation found in the nodular, colloid goiter observed in three out of fourteen hens when the higher dosages of KI were fed. Note the transitional tissue progressing from right to left. The condensed tissue to the extreme left forms the border of a large colloid nodule or cyst. A, B, C, 225 X; D, 75 X.

smallest was 35 x 30 mm in the longest and shortest dimension as compared to 7 x 3 mm in size for glands of normal birds. Some of the nodules (cysts) observed in these goiters were as large as 10 mm in diameter.

*Discussion.* It is difficult to integrate the present finding of a goiterogenic effect of iodide in the normal animal with the iodine-thyroid literature. Morton, Chaikoff and Rosenfeld showed[16] that thyroxine synthesis by thyroid slices *in vitro* was inhibited by the presence of an excess of iodide ions. In later work, Wolff and Chaikoff[8] obtained an inhibition of thyroxine synthesis *in vivo* which could be prolonged by nephrectomy. They suggest that the effect of large doses of inorganic iodide on a hyperactive gland (Graves' disease) is to reduce thyroxine secretion and thus ameliorate the symptoms of hyperthyroidism.[17] One might guess from this work that continued suppression of the thyroxine secretion of the normal gland would lead to

[16] Morton, M. E., Chaikoff, I. L., and Rosenfeld, S., *J. Biol. Chem.*, 1944, **154**, 381.

[17] Wolff, J., and Chaikoff, I. L., *J. Biol. Chem.*, 1948, **172**, 855.

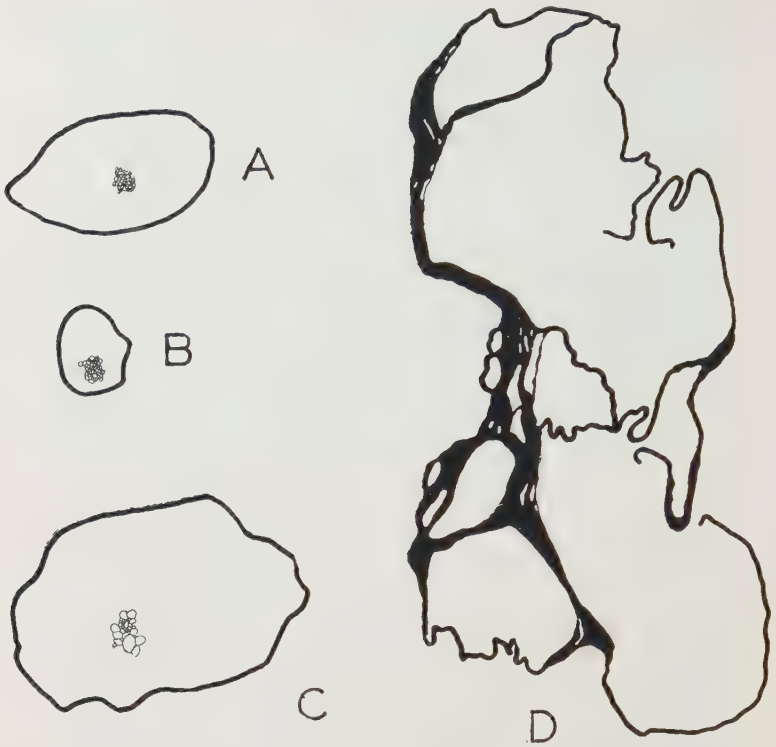


FIG. 3.
Line drawing of median frontal sections of thyroid from: A. Normal hen. B. STP-fed hen. C. KI-fed hen. D. KI-fed hen showing nodular colloid goiter with cystic degeneration. Note the relative follicle size in A, B, C. All figures 4.3 X.

subsequent enlargement of the thyroid via increased thyrotropin from the pituitary.

In the absence of histological evidence this might explain the thyroid enlargement resulting from administration of large doses of iodide. But Rawson *et al.*[18] have differentiated between the antithyroid action of iodide and thiouracil on a hyperplastic gland. Whereas thiouracil produces a columnar epithelium in a gland devoid of colloid, iodide produces an involution of epithelial cells and the storage of colloid. These effects of iodide have been recently confirmed for normal animals by Lesser, Winzler and Michaelson,[19] who also noted that rats fed KI but exposed to cold had more normal appearing glands than treated rats held at room temperature. Thus, in this instance, increased thyrotropin partly reduced the colloid storing effect of iodide. In the light of these findings, it is doubtful if an increase in thyrotropin secretion can adequately explain the goiterogenic effects of iodide, but no alternative hypothesis can be proposed at this time.

*Summary.* The goiterogenic action of increased iodide intake is demonstrated both in the normal fowl, following continuous treatment, as well as in the developing chick embryo. The goiters in chicks presumably result from large amounts of inorganic iodide deposited in the egg by the hen and are comparable in weight and histology to those observed in chicks from dams fed synthetic thyroprotein. It is suggested that the goiters observed in chicks from dams fed synthetic thyroprotein are caused by the iodide present in thyroprotein.

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[18] Rawson, R. W., Moore, F. D., Peacock, W., Means, J. H., Cope, O., and Riddell, C. B., *J. Clin. Invest.*, 1945, **24**, 869.

[19] Lesser A. J., Winzler, R. J., and Michaelson, J. B., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**. 571.

# A Method for Experimental Production of Gradual Occlusion of the Portal Vein.* (17398)

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A sudden complete occlusion of the portal vein is invariably fatal. However, a gradual occlusion of the portal vein in the dog and other laboratory animals is compatible with life, as was demonstrated first by Oré[1] and later by Bernard,[2] Tilmann,[3] Ito and Omi,[4] Neuhof,[5] Boyce *et al.*,[6] Dragstedt,[7] and Brunschwig *et al.*[8] Survival is dependent upon the development of collateral venous channels in the gastrohepatic mesentery, and anastomoses with the esophageal, caval, renal, and rectal veins.

Gradual occlusion of the portal vein has been used by Dragstedt[7] as a substitute for Eck fistula in physiologic studies of the liver and the abdominal organs draining into the portal system. It has also been studied experimentally by Brunschwig *et al.*[8] with a more clinical application in mind. The proximity of the portal vein to the pancreas, and its occasional invasion by pancreatic carcinoma in man, led Brunschwig and his co-workers to investigate methods for the production of adequate collateral channels, thereby enabling excision of this vessel. Menon[9] and Kershner *et al.*[10] attempted to produce portal hypertension in the laboratory animal by gradual occlusion of the portal vein. Consistent increases in portal venous pressure with ascites, esophageal varices, and splenomegaly were not obtained.

All of the methods reported for gradual occlusion of the portal vein were ligature technics with various modifications. Each of the technics required at least a 2-stage operative procedure, with the exception of that described by Brunschwig *et al.*[8] These workers were able to occlude the portal vein successfully by means of a linen thread looped around the vein, the ends of which were brought out through the operative wound and tied over the back of the animal. These ends were pulled upon slightly each postoperative day until the intact loop was pulled out, denoting that the loop had passed through the portal vein. Although gradual occlusion of the portal vein was accomplished successfully by this method, the incidence of fatal peritonitis was high.

The present study is a report of the use of irritative cellophane (Polythene‡) and tantalum to produce gradual portal vein occlusion. It is believed that this method possesses certain advantages over the ligature technics formerly used. It eliminates the repeated operative procedures necessitated by ligation in stages, and also obviates the substantial incidence of peritonitis associated with ligature transection of the portal vein.

*Method.* Healthy adult mongrel dogs were used. Following intravenous sodium pento-

* This project received financial support from the Medical Research and Development Board, Office of the Surgeon General, U. S. Army.

† Life Insurance Medical Student Research Fellow.

1 Oré, *Compt. rend. Acad. Royal d. sc.*, 1856, **43**, 463 (cited by Brunschwig *et al.*[8]).

2 Bernard, C., J. B. Baillière et fils, Paris, 1877, p. 316 (cited by Brunschwig *et al.*[8]).

3 Tilmann, H., *Deutsche med. Wchnschr.*, 1899, **25**, 284.

4 Ito, H., and Omi, K., *Deutsche Z. f. Chir.*, 1902, **62**, 141 (cited by Brunschwig *et al.*[8]).

5 Neuhof, H., *Surg., Gynec. and Obst.*, 1913, **16**, 481.

6 Boyce, F. F., Lampert, R., and McFetridge, E. M., *J. Lab. and Clin. Med.*, 1935, **20**, 935.

7 Dragstedt, L. R., *Science*, 1931, **73**, 315.

8 Brunschwig, A., Bigelow, R., and Nichols, S., *Surgery*, 1945, **17**, 781.

9 Menon, T. B., *J. Path. and Bact.*, 1938, **46**, 357.

10 Kershner, D., Hooton, T. C., and Shearer, E. M., *Arch. Surg.*, 1946, **53**, 425.

‡ Polythene (DuPont), type NV-7-14 with dicetyl phosphate, 1.5 mils in thickness, furnished by The Cellophane Division, E. I. DuPont de Nemours and Company, Wilmington, Del.

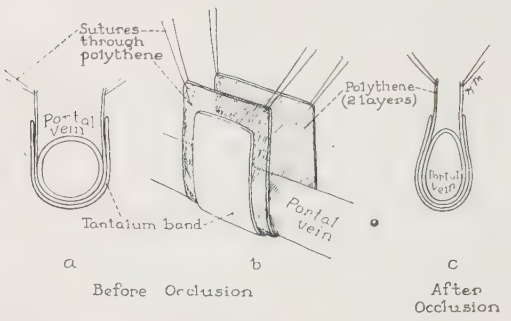


FIG. 1.
Schematic drawing illustrating application of Polythene and tantalum band to the portal vein.

barbital anesthesia, the abdomen was opened through a high right upper rectus muscle-splitting incision. The portal vein was mobilized and a double thickness cuff of Polythene, 1 to 2 cm in length, was wrapped about the vein at the porta hepatis. The Polythene casing was anchored by 2 interrupted No. 00000 arterial silk or cotton sutures. A narrow tantalum band was then placed about the Polythene and tightened so as to decrease the transverse diameter of the portal vein to approximately one-half to two-thirds of the original size (Fig. 1). A cuff of Polythene protruded from beneath the band at either end, and the tantalum did not contact the vein at any point.

After a period of 5 to 10 minutes the small bowel was examined for evidence of dangerously increased venous congestion. If marked venous congestion with cyanosis of the bowel was present, the tantalum band was loosened slightly. The abdominal wall was closed in layers only after the bowel was seen to be normal. A slight distention of the intestinal veins, with approximately a doubling of venous pressure, was usually present. Venous pressures in the portal bed were measured before and after placing of the tantalum band by means of the Burch phlebomanometer or by a spinal fluid manometer.

The animals were either re-explored or sacrificed 3 to 60 days later and the portal vein removed for gross and microscopic examination.

*Results.* The portal vein in 28 dogs was wrapped with Polythene and partially occluded with tantalum. One dog died within 3 hours of the time of operation, and 3 dogs expired within 12 hours. Postmortem examination revealed the characteristic findings of sudden acute portal occlusion. The mesenteric vessels were engorged, the spleen congested, and there were hemorrhagic areas in the mesentery and intestinal wall.

The remaining 24 dogs showed no immediate ill effects from the procedure. The portal vein was excised from 16 of these dogs without a fatality, at intervals ranging from 5 to 60 days (Fig. 2). At the time of the second operation, extensive collateral venous channels were present in the gastrohepatic mesentery. In addition, an increased tendency for the animal to go into shock was noted at the second operation, and was combatted by the use of intravenous fluids and cardiac stimulants. This was an important finding and deserves further study. Two dogs expired following portal vein excision on the fifth postoperative day. The remaining 6 dogs were sacrificed at varying intervals, with the collateral circulation being extensively studied.

Upon gross examination, the wall of the portal vein was thickened, the extent of thickening being dependent upon the length


FIG. 2.
Photograph of excised portal vein with the Polythene and tantalum band in place.

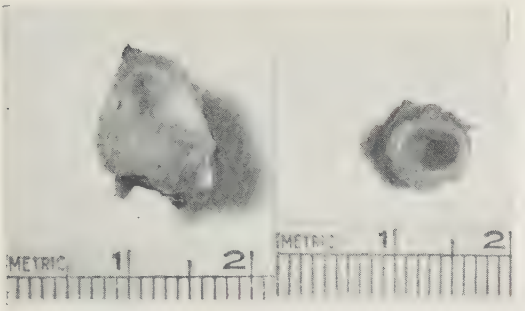

FIG. 3.

*a*. Photograph of excised portal vein segment showing the reaction caused by the application of Polythene 60 days before. Note the absence of lumen and the marked thickening of the vessel walls.

*b*. Normal portal vein segment.

of time during which the vessel had been in contact with the Polythene. The lumen was reduced in all animals and by 30 days after wrapping only a small aperture remained. The lumen was completely obliterated in 4 dogs at 40 to 60 days. Surrounding the vein there was a moderately thick, brown gelatinous pseudomembrane (Fig. 3).

Microscopic examination of the portal vein from animals dying within 12 hours showed a fibrinous reaction with occasional lymphocytic infiltration in the tissues immediately adjacent to the portal vein. At 5 days, changes consisted of edema of the media and adventitia, round cell infiltration, and beginning fibroplasia; in addition, a fibrinous deposition in the tissues adjacent to the vein was noted. Localized hemorrhage into the wall of the vein was occasionally seen. At 10 to 15 days the acute reaction was less evident and the significant finding was medial, adventitial, and periportal fibrosis. At 18 to 20 days there was increased fibrosis and chronic inflammatory response in the outer half of the vein wall and periportal tissues. After a period of 30 to 60 days, intimal thickening with marked fibrosis of the media and adventitia was present (Fig. 4). Apparently the degree of fibroplasia was dependent upon the duration of Polythene application. It was also noted that the chronic inflammatory response was significantly decreased after 30 days.

One of the original objectives of the study was to attempt to produce portal hypertension by this method. However, at the time of re-exploration there was no elevation of venous pressure in the intestinal veins. Extensive collateral channels joined the renal, rectal, and caval veins, and were present in the gastrohepatic omentum. In some instances gastrohepatic collateral vessels were larger than the portal vein prior to Polythene and tantalum wrapping. The number and size of the collateral vessels was in direct proportion to the duration of portal occlusion. Injection studies of the venous collaterals, using liquid latex and Hill's media, revealed no instance of a collateral vessel passing directly into the liver. Significant anastomoses with esophageal veins were not demonstrated. This is of interest when compared with the usual collateral channels found in man following blockage of the portal vein.

*Discussion.* Irritative cellophane has been used in the treatment of aneurysms,[11,12,13] the experimental production of aortic stenosis and obliteration,[14,15] and in attempts to obliterate a patent ductus arteriosus.[16] The dicetyl phosphate present in the Polythene is responsible for the irritative properties of the material,[17] and differentiates this cellophane from the non-irritating type which does not induce fibroplasia and which has been used in certain orthopedic procedures.[18,19] We have not encountered any reports in the literature regarding the use of irritative cellophane in occlusion of major veins. It has

11 Harrison, P. W., and Chandy, J., *Ann. Surg.*, 1943, **118**, 478.

12 Poppe, J. K., and De Oliveira, H. R., *J. Thoracic Surg.*, 1946, **15**, 186.

13 DeTakats, G., and Reynolds, J. T., *Surgery*, 1947, **21**, 443.

14 Pearse, H. E., *Ann. Surg.*, 1940, **112**, 923.

15 Cooper, F. W., Jr., Robertson, R. L., Shea, P. C., Jr., and Dennis, E. W., *Surgery*, 1949, **25**, 184.

16 Harper, F. R., and Robinson, M. E., *Am. J. Surg.*, 1944, **64**, 294.

17 Yeager, G. A., and Cowley, R. A., *Ann. Surg.*, 1948, **128**, 509.

18 Wheeldon, T., *J. Bone and Joint Surg.*, 1939, **21**, 393.

19 McKeever, D. C., *J. Bone and Joint Surg.*, 1943, **25**, 576.

a. b.

FIG. 4.

*a*. Photomicrograph of a segment of portal vein 30 days after the application of Polythene and tantalum (× 25). Note the marked fibrosis and chronic inflammatory response in the media and adventitia.

*b*. Photomicrograph of a normal portal vein segment (× 25).

been stated that the aorta of the dog may be occluded with Polythene and tantalum in 12 weeks.[15] The interval required for total occlusion of the portal vein was approximately 6 to 8 weeks and the tissue response of the vein was similar to that described for the aorta.

The 4 dogs which died within 12 hours of operation illustrate the difficulty in securing maximal immediate constriction without occluding the vein to a point incompatible with life.

The shortest time interval compatible with survival between partial ligature occlusion of the portal vein and excision or complete ligation of this vessel has been reported as 10 days.[6,8] Four of 6 dogs in this series which were subjected to excision 5 days after partial occlusion survived. This suggests the development of a more extensive collateral venous network with the present method of occlusion, and clinically may be important in preparing for elective ligation of the portal vein.

It is believed that the gradual fibroplastic obliteration of the portal vein produced by the irritating cellophane (Polythene) more closely approximates the gradual reduction of portal blood flow as seen in certain disease states, *e.g.* the cirrhotic diseases, and will facilitate the study of these diseases.

*Conclusions*. 1. The fibroplastic response in the outer half of the vein wall and in the periportal tissues is similar to the reaction seen in the aorta following application of Polythene and tantalum.

2. At least 6 to 8 weeks are required for complete occlusion of the portal vein by Polythene-induced fibroplasia.

3. The gradual occlusion of the portal vein produced by irritative Polythene may be valuable in preparing for elective ligation of the portal vein and in the experimental study of decreased portal blood flow.

*Summary*. A one stage procedure for gradual occlusion of the portal vein using an irritative type of cellophane (Polythene) and tantalum is described.

The portal vein in 28 dogs was wrapped with Polythene and partially occluded with a tantalum band. The veins were removed for gross and microscopic examination 3 to 60 days later. Extensive venous collateral channels developed in the gastrohepatic mesentery and along the posterior peritoneum, passing into the renal, rectal, and caval veins. In 4 of 6 dogs venous anastomoses were adequate to sustain life following excision of the portal vein 5 days after partial portal occlusion.

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## Neutralization of Three Immunological Types of Poliomyelitis Virus by Human Gamma Globulin.* (17399)

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Recent studies of antigenic groups of poliomyelitis virus have shown the existence of at least 3 distinct types.[1,2] Two of these are widespread in their distribution, but the third is represented thus far by only one strain (Leon) isolated in Los Angeles, California in 1937.[3] In an attempt to determine whether this strain is perhaps unusual in its occurrence, or is sufficiently widespread to be considered of importance in the epidemiology of this disease, neutralization tests with human gamma globulin were caried out using this strain as well as representatives of the other two antigenic types, Lansing and Brunhilde.[1]

*Methods.* The viruses used have been described in detail before,[1] and the pools used are designated as Brunhilde III, Lansing VIII, and Leon I. These virus pools are of high titer, and consist of 10 or more cords of rhesus monkeys killed on the first day of paralysis. Only lumbar and cervical enlargements were used. Aqueous suspensions were prepared with a Waring blendor, and aliquots sealed in glass ampules and stored on dry ice. Titrations of these pools are shown in Table I.

The globulin solution used was a sample supplied by the courtesy of the American National Red Cross who distribute it for measles prophylaxis. The sample used was prepared by E. R. Squibb and Sons, and information regarding it was kindly supplied by Dr. J. W. Palmer of the Squibb Company. The human plasma pool used in the preparation of the globulin pool from which this sample was derived totalled 3,000 liters and consisted of the surplus plasma returned to the American Red Cross by the armed forces after the war. The plasma is therefore representative of about 20,000 to 50,000 individuals who contributed blood to the Red Cross during the war. These individuals lived predominantly on the East Coast and in the Great Lakes Area. A small fraction of the plasma was also collected in the Far West. The plasma was originally dried and subsequently reconstituted. The primary fractionation was made following method 6 of Cohn, *et al.*;[4] the subfractionation was made according to method 9 of Oncley, *et al.*[5] The preparation of the globulin fraction from plasma does not appear to result in deterioration of antibody.[6] The effect of prolonged storage of the plasma in the dried state might conceivably have had a detrimental effect on antibody levels, although this seems doubtful in view of the

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

1 Bodian, D., Morgan, I. M., and Howe, H. A., *Am. J. Hyg.*, 1949, **49**, 234.

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TABLE I.
Titrations of Virus Pools Used.

| Virus and pool No. | Dilutions of cord suspensions in water | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | PD50* |
| Brunhilde III | | 24/24† | 24/25 | 14/14 | 4/6 | 2/6 | 0/6 | $10^{-5.6}$ |
| Lansing VIII | 9/9 | | 13/14 | 4/6 | 3/6 | 0/6 | | $10^{-4.7}$ |
| Leon I | 15/15 | 8/8 | 16/16 | 8/8 | 8/8 | 6/10 | 3/10 | $10^{-6.3}$ |

* PD50 calculated with paralytic rates taken on a per cent basis.
† Number paralyzed over number inoculated (accumulated inoculations).

TABLE II.
Neutralization of Poliomyelitis Type Viruses by Human Gamma Globulin.

| | Viruses (100 PD50) | Globulin dilutions | | | NMS* |
|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | |
| A. 6/7/49 | Brunhilde | 0/2† | 1/2 | 2/2 | 3/3 |
| | Lansing | 0/2 | 0/2 | 2/2 | 3/3 |
| | Leon | 0/2 | 0/2 | 2/2 | 2/3 |
| B. 8/24/49 | Brunhilde | 0/2 | 2/4 | 2/2 | |
| | Lansing | 0/1 | 1/3 | 2/2 | |
| | Leon | 0/2 | 0/4 | 2/2 | |

| | | Globulin dilutions | | | | Neutralization index§ |
|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | PD50‡ | |
| Total A + B | Brunhilde | 0/4 | 3/6 | 4/4 | $10^{-2}$ | 10,000 |
| | Lansing | 0/3 | 1/5 | 4/4 | $10^{-2.4}$ | 25,000 |
| | Leon | 0/4 | 0/6 | 4/4 | $10^{-2.5}$ | 30,000 |

* Normal monkey serum.
† Number monkeys paralyzed over number inoculated.
‡ Calculated by the method of Reed and Muench.[8]
§ Defined here as the antilog of the sum of the exponents of the PD50 of the virus used and the PD50 dilution of globulin. The neutralization index is thus based on the titer of the virus in aqueous solution, and the assumption is made that virus dilutions and globulin dilutions are equivalent.

results to be described.

Our sample consisted of 2 ml of a solution containing 16.8% of gamma globulin, as compared to 0.74% contained in normal plasma, so that the gamma globulin is not only purified but also concentrated to about 23 times the level of normal plasma. It should contain most of the antibodies of adult plasma. The original solution used in the tests to be described was diluted with physiological saline to prepare 1 to 10, 1 to 100, and 1 to 1000 dilutions. These were used immediately for the first series of neutralization tests and the remaining solution stored at 4° C until used in the repeated tests.

The diluted globulin solution was mixed in equal proportions with virus suspensions to prepare the material for inoculation. The mixtures contained 100 PD50 of virus in 0.4 ml, in each instance. The mixtures were allowed to stand at room temperature for 2 hours, and then at 4° C for 2 hours, before inoculation. Each monkey was inoculated into the left thalamus with 0.4 ml of the globulin-virus mixture according to the method described in detail elsewhere.[7]

All monkeys were observed for 3 weeks for signs of poliomyelitis. Animals which did not show typical signs, including severe paralysis, were then prepared for histopathological examination of brain and spinal cord. The results recorded were based on the results of these procedures. No instances of non-paralytic poliomyelitis were revealed by the

[7] Bodian, D., Morgan, I. M., and Schwerdt, C. E., *Am. J. Hyg.*, 1950, **51**, No. 1.

[8] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

histological studies.

*Results.* Results are shown in Table II. They indicate that the neutralizing capacity of gamma globulin in this sample is very high for each of the 3 antigenic types of poliomyelitis virus. The results of the two sets of tests, made with separate virus aliquots, show agreement and indicate that the concentrated gamma globulin can neutralize about 30,000 PD50 of the Lansing and Leon viruses, assuming that virus and globulin dilutions are comparable. Storage of the globulin solution at 4° C for almost 3 months does not appear to have impaired the neutralizing potency.

*Discussion.* The most interesting result of these tests is that pooled adult gamma globulin may contain as much antibody against Leon virus, as against the representatives of the other two types, which are known to be widespread in distribution.[1,2] Since relatively few strains have thus far been differentiated as to type, it is possible that more representatives of the Leon type will be isolated in the future. The question may be raised as to whether this type may have been more or less restricted to the Far West, where Leon was isolated in 1937, especially since our sample of gamma globulin contained some material from that area. In view of the fact that only a small fraction of the plasma pool was obtained from individuals in the Far West, it is doubtful that the Leon antibody was derived only from this source. Since the titer of Leon antibody was equal to that of the Lansing and superior to the Brunhilde virus, there seems every reason to suppose that the Leon virus type is, or was, of importance in the epidemiology of poliomyelitis. It is of further interest that antibody against Brunhilde virus, the representative of the type currently found to be most prevalent,[1] seemed to be present in somewhat less concentration than antibody against the other two types. It is obvious, however, that this preliminary study should be extended to determine in greater detail not only the geographical distribution of type-specific antibodies in human sera, but also the distribution in time and in various age groups. It is also possible to speculate that viruses of the Brunhilde type, although more prevalent than those of other types, may be inferior as antigens.

Antibody against Lansing poliomyelitis virus has previously been demonstrated in human gamma globulin.[6,9] The fact that antibodies against all 3 known antigenic types of poliomyelitis virus exist in high concentration in gamma globulin adds further emphasis to the report of Bahlke and Perkins,[10] who found that relatively large amounts of this material were ineffective in the treatment of preparalytic poliomyelitis. Although elaborate controls were exercised in their study, and although it is now clear that high polyvalent antibody levels are present in gamma globulin, their conclusion that serum therapy, in any form, is ineffective in poliomyelitis still seems to require some qualification. First, the virus, or viruses, responsible for the cases they attempted to treat was not identified as to type, so that a remote possibility exists that specific antibody was not present in the gamma globulin used. Moreover, unconcentrated hyperimmune monkey serum contains levels of antibody approximately the same as those in human gamma globulin.[11,12] Preparation of gamma globulin from hyperimmune serum could therefore be expected to produce antibody levels about 20 times greater than those found in human gamma globulin.

*Summary.* A sample of gamma globulin, refined and concentrated about 23-fold from pooled adult human plasma, was tested for neutralizing antibodies against representatives of three distinct antigenic types of poliomyelitis virus, Brunhilde, Lansing, and Leon. The neutralizing potency was high against all three viruses. A dilution of the globulin solution of 1/100 neutralized 100 PD50 of virus in almost every instance. A dilution of 1/1000 failed to neutralize 100 PD50 of virus.

[9] Kramer, S. D., quoted in [10].

[10] Bahlke, A. M., and Perkins, J. E., *J.A.M.A.*, 1945, **129**, 1146.

[11] Morgan, I. M., *J. Immunol.*, 1949, **62**, 301.

[12] Howe, H. A., *Am. J. Hyg.*, in press.

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# Pteroylglutamic Acid, Ascorbic Acid, and Injectable Liver Extract on Dietary Glycine Toxicity in the Rat.* (17400)

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Previous work from this laboratory has demonstrated that the growth retardation resulting from inclusion of sodium benzoate in a purified diet for rats may be in part overcome by the addition of pteroylglutamic acid (PGA) in the diet.[1] Experiments were next designed to study the effect of feeding high glycine diets to rats. In confirmation of the work of Hier *et al.*[2] it was found that rats receiving a purified diet containing 10% glycine grew at a subnormal rate; hence the effects of supplementing this diet with PGA, injectable liver extract, and ascorbic acid were studied. While these experiments were in progress Martel and Gingras[3] reported that the feeding of a 10% glycine diet to rats resulted in growth suppression, mild leucopenia, and an elevated creatinuria, all of which responded to PGA.

*Experimental.* Six groups of weanling Sprague-Dawley rats, 4 males and 4 females in each group, were fed the diets given in Table I. The rats were fed *ad libitum* and food intake was measured. Complete blood counts were made on the 37th and 46th days of the experiment. The differences in weight gains among the various groups were statistically treated by the analysis of variance method.[4]

*Results and Discussion.* The growth curves are given in Fig. 1. It may be observed that the inclusion of 10% glycine in the purified diet resulted in a greatly decreased rate of growth. PGA markedly improved the growth of rats receiving the high glycine diet. The addition of liver extract to the high glycine diet resulted in some growth improvement but less than that produced by PGA. The liver extract used was assayed for PGA and found to contain 2.62 $\gamma$ per cc; this amounted to approximately 0.2 $\gamma$ of PGA per rat per day. This and the fact that the group receiving PGA plus liver extract grew no faster than those receiving PGA alone would indicate that some of the growth improvement resulting from the liver extract may have been due to its content of PGA.

Johnson and Dana[5] have presented data to indicate that ascorbic acid increased the rate of growth and induced a blood response in rats fed sulfasuxadine. Woodruff and Darby[6] have reported that the inclusion of PGA in the diet of scorbutic guinea pigs reduced the excretion of tyrosine metabolites. Such data indicate a similarity in function of PGA and ascorbic acid and for this reason one group of rats was fed the high glycine diet plus ascorbic acid. It may be seen from the growth curves that ascorbic acid had little effect on the growth of the rats. The difference in rate of gain between the group receiving ascorbic acid and the group receiving high glycine alone was not statistically significant.

Weight gains per gram of food eaten were calculated for the various groups and the average values were: control 0.295, high glycine 0.152, high glycine plus PGA 0.282, high glycine plus liver extract 0.205, high

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1 Totter, John R., Amos, Esther S., and Keith, Cecilia K., *J. Biol. Chem.*, 1949, **178**, 847.

2 Hier, Stanley W., Graham, Claire E., and Klein, David, Proc. Soc. Exp. Biol. and Med., 1944, **56**, 187.

3 Martel, F., Page, E., and Gingras, R., *Rev. Canadienne de Biologie*, 1947, **VI**, 802.

4 Snedecor, George W., Statistical methods applied to experiments in agriculture and biology, The Iowa State College Press, 4th ed., 1946.

5 Johnson, B. Connor, and Dana, Ann S., *Science*, 1948, **108**, 210.

6 Woodruff, Calvin W., and Darby, William J., *J. Biol. Chem.*, 1948, **172**, 851.

TABLE I.
Composition of Experimental Diets.

| Dietary component | Groups I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| Casein, % | 18 | 18 | 18 | 18 | 18 | 18 |
| Sucrose, % | 75 | 65 | 65 | 65 | 65 | 65 |
| Crisco, % | 3 | 3 | 3 | 3 | 3 | 3 |
| Cod liver oil, % | 2 | 2 | 2 | 2 | 2 | 2 |
| Salt mix* % | 2 | 2 | 2 | 2 | 2 | 2 |
| Glycine, % | 0 | 10 | 10 | 10 | 10 | 10 |
| PGA—mg/100 g | 0 | 0 | 0.5 | 0 | 0.5 | 0 |
| Liver extr.—cc/kilo | 0 | 0 | 0 | 8 | 8 | 0 |
| Ascorbic acid—mg/100 g | 0 | 0 | 0 | 0 | 0 | 100 |

The liver extract was an anti-pernicious anemia concentrate containing 15 units per cc. The following vitamins were added per 100 g to all diets:

| | mg | | mg |
|---|---|---|---|
| Thiamine | 0.5 | Choline chloride | 10.0 |
| Riboflavin | 0.5 | Pyridoxine hydrochloride | 0.5 |
| Nicotinic acid | 2.0 | Biotin | 0.005 |
| i-Inositol | 10.0 | Vit. K | 0.025 |
| Calcium pantothenate | 1.0 | | |

* Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, 1937, **14**, 273.

glycine plus PGA plus liver extract 0.291, and high glycine plus ascorbic acid 0.213. With the exception of reversal between the ascorbic acid and liver extract groups, efficiency of gain ranked the same in the various groups as did total weight changes.

The hematological data are presented in Table II. Data for males and females are given separately only in Groups IV and V, since there were no sex differences in the other groups. The feeding of the high glycine diet did not produce a significant leucopenia in these rats when compared to the controls; however, addition of PGA to this diet did elevate the white cell count. Addition of liver extract and liver extract plus PGA to the high glycine diet resulted in an increased white count in male rats but was without effect in the females.

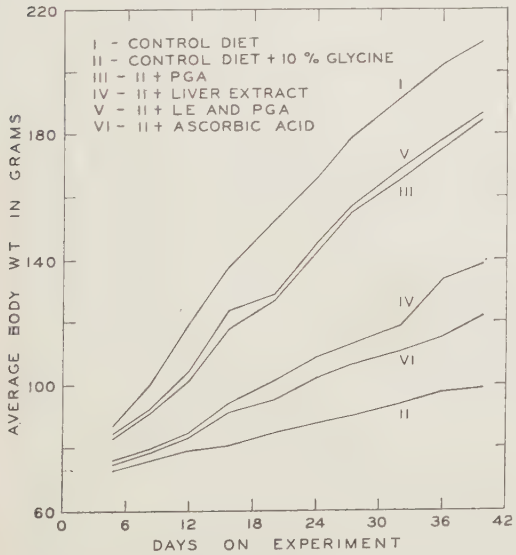


FIG. 1.
Growth curves for rats receiving the various diets.

Although the red cell counts of rats receiving the high glycine diet were lower than the control group, none of the supplements tested were effective in restoring the counts to the level of the controls. Hemoglobin values were also reduced by the high glycine diet and were improved only by the addition of the combination of PGA plus liver extract or by ascorbic acid. There were no consistent differences in hematocrit or differential white cell count among the various groups. The rats receiving ascorbic acid were found to have a higher mean corpuscular volume than any of the other groups. It may also be noted that rats receiving ascorbic acid had the lowest red count of any of the groups, but had a higher hemoglobin level than any of the others except the control group and the

TABLE II.
Average Hematological Data for Rats Receiving the Various Diets.

| Group | Wbc., $10^3/mm^3$ | Reticulocytes, % of rbc. | Granulocytes, % of wbc. | Lymphocytes, % of wbc. | Rbc., $10^6/mm^3$ | Hb., g/100 cc | Vol. packed Rbc. cc/100 cc | Mean corpuscular vol., microns$^3$ |
|---|---|---|---|---|---|---|---|---|
| I. Control | 17.9 | 2.6 | 11 | 89 | 7.29 | 14.9 | 51 | 70 |
| II. High glycine | 16.4 | 2.2 | 17 | 83 | 6.75 | 13.9 | 49 | 74 |
| III. High glycine + PGA | 19.1 | 2.2 | 10 | 90 | 6.70 | 13.2 | 49 | 74 |
| IV. High glycine + L.E. | | | | | | | | |
| ♂ | 20.7 | 2.3 | 20 | 80 | 6.29 | 13.1 | 46 | 73 |
| ♀ | 14.5 | 2.5 | 12 | 88 | 6.09 | 13.9 | 49 | 81 |
| V. High glycine + PGA + L.E. | | | | | | | | |
| ♂ | 26.5 | 3.5 | 17 | 83 | 6.80 | 14.9 | 51 | 75 |
| ♀ | 17.5 | 3.6 | 9 | 91 | 6.27 | 14.6 | 50 | 79 |
| VI. High glycine + ascorbic acid | 16.4 | 3.1 | 13 | 87 | 6.20 | 14.1 | 50 | 82 |

group receiving liver extract plus PGA.

There is not sufficient evidence available at present to permit an explanation of the growth-promoting effect of PGA when added to a high glycine diet for rats. Holland and Meinke[7] have presented evidence which indicates that PGA may function in the synthesis of serine by *Streptococcus faecalis*. It has been shown by Sakami[8] that glycine is converted to serine and then to glycogen by the rat. It is possible that this conversion may be a factor in explanation of our results. Experiments are in progress to study the effects of PGA on the conversion of glycine to serine by liver homogenates.

*Summary.* Weanling albino rats were fed a standard purified diet, and a similar diet containing 10% glycine replacing an equal amount of sucrose. Groups of rats were given the glycine-containing diet supplemented respectively with PGA, injectable liver extract, PGA plus liver extract, and ascorbic acid. The growth of rats receiving the high glycine diet was greatly reduced as compared to the control group. Supplementation of the high glycine diet with PGA and with PGA plus liver extract resulted in a marked improvement in growth rate; liver extract alone was less effective; and ascorbic acid did not significantly improve rate of growth of rats receiving the 10% glycine diet. Rats receiving the high glycine diet did not develop a marked leucopenia when compared with control rats; however, supplementation of this diet with PGA resulted in an increased white count while supplementation with liver extract and with liver extract plus PGA resulted in an increased white count in male rats but was without effect in females. The mean corpuscular volume was found to be highest in rats receiving the high glycine diet plus ascorbic acid.

[7] Holland, Bryant R., and Meinke, W. W., *J. Biol. Chem.*, 1949, **178**, 7.

[8] Sakami, W., *J. Biol. Chem.*, 1948, **176**, 995.

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# The Effect of Staphylococcal Enterotoxin upon the Frog, (*Rana pipiens*). (17401)

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Significant advances in the study of staphylococcal food poisoning have been extremely difficult because of the lack of a reliable diagnostic test. Bioassay methods have been described using human volunteers, monkeys, suckling pigs, and kittens and cats, [1–4] none of which has proved to be entirely satisfactory. The ability of an enterotoxin produced from a strain of *Micrococcus pyogenes var. aureus* to elicit a vomit reaction in a test animal has been considered evidence of a positive test. Animals such as ruminants, horses, and rodents lack the appropriate coordinating vomit mechanism[5] and hence the usual laboratory animal (rabbit, guinea pig, rat, and mouse) has been of little value. The common frog, *Rana pipiens,* was chosen for this study because it has the ability to exhibit reverse peristalsis of the stomach,[6] is readily available, is inexpensive, and is easily maintained under laboratory conditions.

*Procedures and results.* Preliminary experiments were carried out, using a filtrate prepared from a known enterotoxigenic strain of *Micrococcus pyogenes var. aureus.** Dolman's[7] technique was followed in detail in the preparation of this and all subsequent filtrates. Only healthy, well-nourished frogs were used. Filtrates were first administered either parenterally (into the dorsal lymph sac) or orally in No. 00 gelatin capsules. Satisfactory results were obtained, however, by the simpler procedure of direct feeding with a medicine dropper. This was accomplished by opening the frog's mouth with large, blunt-ended forceps, and then, while holding the mouth open by a thumb inserted near the angle of the jaw,[8] slowly inserting the dropper through the esophageal sphincter. Thus, the filtrate was placed directly into the frog's stomach. The frog under test was placed in an aquarium jar containing a small amount of water and was observed over a period of hours. A positive test for enterotoxin is shown by the production of a series of reactions termed for the purpose of this discussion a "spasm", for although reverse peristalsis of the stomach could be demonstrated, vomiting of the stomach contents never occurred.

A spasm is initiated by a series of swallowing motions (retraction of the eyeballs), a slowly repeated gaping of the mouth, a gradual change in the tone of the skin color, a distension of the abdominal wall, a distinctive sitting position (the forelegs at full height and the back raised so that it parallels the floor of the aquarium). This takes place within 3 to 10 minutes and reaches a climax when the spasm is completed by a slow-motion rubbing of the abdominal wall with a hind-foot followed by a wiping of the face and mouth with a fore limb. This occurs on both sides of the body and may last from 3 to 15 minutes. Gradually, mucous is expelled from the mouth, abdominal distension is decreased and the frog assumes a natural sitting position. Gaping of the mouth may continue for some time after the other characteristic body motions have stopped. The rapidity with which the spasm follows the

1 Jordan, E. O., *J.A.M.A.*, 1931, **97**, 1704.

2 Jordan, E. O., Proc. Soc. Exp. Biol. and Med., 1931, **29**, 161.

3 Hopkins, E. W., and Poland, E. F., *Food Res.*, 1942, **7**, 414.

4 Dolman, C. E., *Canad. J. Pub. Health,* 1926, **27**, 489.

5 Meyer, H. H., and Gottlieb, R., *Experimental Pharmacology,* J. B. Lippincott, Philadelphia, Pa., 1926, 2nd ed.

6 Alvarez, W. C., *An Introduction to Gastroenterology,* P. B. Hoeber, N. Y., 1940, 3rd ed.

7 Dolman, C. E., *Canad. J. Pub. Health,* 1940, **51**, 68.

* Strain No. 8 obtained through the kindness of Dr. Gail Dack, Department of Bacteriology and Parasitology, University of Chicago.

8 Rose, S. Meryl, personal communication, 1949, Department of Zoology, Smith College.

feeding of the filtrate is apparently dependent upon individual susceptibility and enterotoxin potency. Reactions have been observed within a range of 45 minutes to 7 hours. After the spasm has subsided, further reactions may be elicited, for at least a 10-day period, by feeding the frog. Therefore, it is possible to feed and then leave the animal until the next morning when a meal is given. A typical spasm occurs within 3 to 4 hours.

Such typical reactions have been obtained with other known enterotoxigenic strains of *Micrococcus pyogenes var. aureus,*† while no such reaction has ever been observed in frogs fed with filtrates prepared from known negative strains of *Micrococcus pyogenes var. aureus* No. 902 and 64, *Escherichia coli, Porteus vulgaris,* and sterile culture medium. The results of these tests are summarized in Table I.

TABLE I.
Effect of Enterotoxigenic and Non-Enterotoxigenic Filtrates Upon the Frog (*Rana pipiens*).

| Organism used in filtrate preparation | Total No. of frogs tested | Reaction observed |
|---|---|---|
| *Micrococcus pyogenes var. aureus* | | |
| No. 161 | 28 | Spasms |
| No. 8 | 8 | ,, |
| No. 432 | 21 | ,, |
| No. 422 | 5 | ,, |
| No. 64 | 18 | None |
| No. 902 | 5 | ,, |
| *Micrococcus pyogenes var. albus* | 9 | ,, |
| *Escherichia coli* | 6 | ,, |
| *Proteus vulgaris* | 3 | ,, |
| Sterile culture medium | 29 | ,, |

A series of frogs was fed known enterotoxigenic filtrates in an effort to show reverse peristalsis of the stomach. At the height of a spasm the animal was decapitated. In each frog studied, the esophageal sphincter was relaxed and the stomach was observed in reverse motion. Controls included unfed frogs, those fed hamburg, and those fed sterile culture medium. One each of these was sacrificed with each test frog. In no control was any reverse motion of the stomach observed.

*Summary.* Staphylococcal enterotoxins which gave positive results with the kitten test were capable of producing spasms when fed to frogs. It would appear from the results summarized in Table I, that the frog test was specific for no false positive reactions were noted. It is not possible to use a frog more than once when a positive reaction has been obtained and further study is in progress to determine the nature of the injury to the digestive mechanism.

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† Enterotoxigenic strains Nos. 432 and 422 and non-enterotoxigenic strains Nos. 902 and 64 were obtained through the kindness of Dr. Glenn Slocum, Food and Drug Administration, Washington, D.C.

## Epilation in the Non-Irradiated Member of Parabiotically United Rats.* (17402)

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Despite the lack of our ability to explain this phenomenon—the loss of hair in the non-irradiated members of parabiotically united rats—it was thought worthy of record as an observation.

The relatively few known fundamental reactions involved when either particulate or electromagnetic radiation reacts with matter are described in the standard textbooks of Atomic Physics. These explanations are fully

* This work was supported in part by the Atomic Energy Commission.

† Atomic Energy Commission Postdoctorate Research Fellow.

as applicable to animate as to inanimate objects. Nevertheless much is unknown concerning the chain of events which leads to the eventual dissolution or loss of the integrity of a system.

Specific agents are known which produce in a mammal a syndrome comparable to that seen following radiation. For example, it is possible with folic acid antagonists to produce epilation, lymphopenia, anemia, hemorrhage, diarrhea, anorexia, malaise, wasting and eventual death in much the same sequence as occurs with radiation. This can be done with concentrations of these substances so low in body fluids that they avoid chemical detection.

It is possible that the indiscriminate production of ion pairs and excitation of atoms in animals by means of radiation may be followed by the recombination of ions in such a manner that the probability of the formation of a specific substance causing the sequence of events seen in radiation illness is present. Evidence for toxic recombinations has been gained from the study of the radiation of water *in vitro*.[1,2] Other simple *in vitro* experiments have led to the idea of direct and indirect action.[3] A direct action is one in which ionization produced within the molecule causes the effect while an indirect action is one in which the effect occurs because of ionization produced in surrounding material. Apart from this consideration of whether a toxic substance is produced directly or indirectly, a second, perhaps less fundamental question has concerned investigators: *Is the toxic material during and after formation free to move from one part of the animal's system to another and act there?*

The conviction that the production of a specific harmful substance could not occur without being conveyed throughout the animal's system has led to cross circulation experiments in which the vascular flow of a non-treated animal was connected to that of a radiated animal. [4–8] In general these experiments have been performed with complete disregard for the magnitude of the vascular exchange or with some regard but little evaluation. It is particularly important to realize that negative results of cross circulation radiation experiments are worthless so far as disproving an hypothesis that radiation damage is caused by a circulating agent. Moreover similar physical considerations apply to transfer within a single animal. A physico-mathematical consideration of cross circulation experiments has been presented in which it is shown that the distribution of a substance within a system is a function of at least two turnover rates, (1) that of the blood flow to the area and (2) that of the metabolite.[9]

Early investigators had much difficulty with the parabiotic technique—indeed some of them admitted to more operative failures than successes and with a successful operation there was always the question of the efficacy of the inosculation. Zacherl irradiated parabiotic rats with a lethal and unspecified amount of X-ray.[4] He made no statement concerning the specific nature of the shielding afforded the protected members of the pair. He observed concomitant fall in the temperature and white blood count of the members of the pair; however, the fall of both the white blood count and the temperature was not as great in the protected member. He gave little consideration to the effect of dilution which would occur with mixing. He was convinced of a radiation toxin. Behnes' experiments on parabiotic guinea pigs are less clearly described.[5] Woenckhaus also was convinced that a radiation toxin was produced, but his

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2 Fricke, H., *J. Chem. Phys.*, 1934, **2**, 556.

3 Lea, D. E., *Action of Radiations on Living Cells*, chap. 2, Macmillan Co., 1947.

4 Zacherl, H., *Beitrag zür Allgemeinwirkung der Röntgenstrahlen Strahlentherapie, Band*, 1926, **23**, 272.

5 Behne, *Deutsche Med. Wochensch.*, 1920, **46**, 223.

6 Woenckhaus, E., *Arch. f. Exp. Path. und Pharmak.*, 1930, **150-152**, 183.

7 Lawrence, J. S., Dowdy, A. H., and Valentine, W. N., Atomic Energy Commission Declassified Document No. MDDC-853, 1947.

8 Barnes, W. A., and Furth, O. B., *Am. J. Roent.*, 1943, **49**, 662.

9 Huff, R. L., Trautman, R., and Van Dyke, D. C., to be published.

experiments are subject to the same criticism as Zacherl's.[6] Lawrence *et al.*[7] reviewed the literature concerning the evidence for a circulating radiation toxin and concluded that the question was not settled. They set out to confirm or deny the presence of such a substance by cross-circulating cats (carotid to carotid anastomoses) at various time periods following radiation of one with 1500 r X-ray for a duration of approximately 10 hours. In spite of the fall in absolute lymphocyte count which occurred during and after the cross circulation in the non-radiated member they concluded that their results did not support the theory of a radiation toxin. Exact interpretation of their experiments requires that the turnover rates of white cells and of the blood flow between the animals be known. Furth and Barnes[8] performed an extensive experiment on parabiotic mice which included much microscopic tissue examination. The non-radiated members of the pairs showed non-specific changes which were similar to but much less than in the radiated animal. They mentioned that the parabiotic combination offered protection from radiation.

In this laboratory an experiment was planned to determine the magnitude of the protection from radiation afforded rats in parabiosis. During the course of this study it was noted that both the radiated and non-radiated members of a pair showed epilation; starting about 4 weeks after radiation and lasting 6 weeks.

*Methods.* The apparatus used for radiation is shown in Fig. 1. It consisted of a lead chamber in juxtaposition with a lucite chamber between which was a lead divider except in the area corresponding to the connecting tissue of the pairs. The dosage given was determined by inserting a lucite phantom, which has approximately the x-ray absorption equivalent of tissue. Victoreen ionization chambers were placed in holes drilled in the center of the phantom, and a third was placed in an attached lead box to serve as a monitor. Radiation conditions were 200 K.V., 15 ma, 0.5 mm Cu and 1.0 mm Al filters, 37.5 cm tube distance, rate 40 r/min. The time necessary to deliver the desired dose was determined with the ionization chambers in the plastic

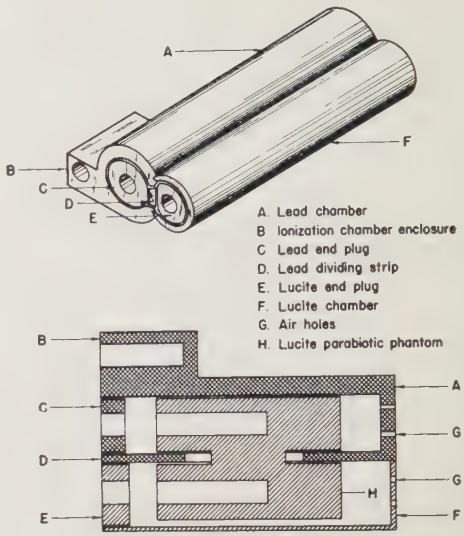


FIG. 1.
Chamber for exposing parabiotic rats to radiation.

phantom. The reading in the lead box was found to be a constant fraction of the dose received inside the phantom, and this reading was used later to check the dose given the animals. The dose delivered inside the phantom protected by the lead chamber was found to be 3% of that given the unprotected member. Readings were made inside the lead chamber by means of the Victoreen Ionization Chambers and also with x-ray film to insure the absence of large amounts of soft radiation from scatter.

Three groups of animals were prepared for the experiment. Group I consisted of 6 parabiotic pairs which were subjected to radiation, Group II was 6 parabiotic pairs not radiated and serving as controls to parabiosis and Group III was composed of 12 single animals radiated in the same manner as those of Group I. All animals were of approximately the same weight so as to insure comparable depth dose. The rats were females of the Slonaker strain which had been propagated by brother and sister inbreeding for 50 generations. This degree of inbreeding should result in an almost homozygous genetic pattern which would tend to eliminate the antigen specificity of their nuclear proteins. Pairs prepared from these animals remain in perfect health for the normal life

expectancy. In none of them is observed the wasting and anemia so often described for parabiotic rats. The vascular exchange rate is .6% of the blood volume per minute.[10] Litter mates were surgically united (coelio-anastomosis) at 20-30 days of age.

After the pairs of Groups I and II had been joined for 2 months the members of Groups I and III were radiated alternately on the same day. Precisely: one pair was placed in the chamber, one animal receiving 27 roentgens and the other 900 roentgens; then 2 single animals were placed in the container one receiving 27 roentgens and the other 900 roentgens, etc. Following radiation 2 animals from each of the 3 groups were maintained in the same cage. Treatment with DDT was carried out weekly.

*Results.* For the most part the protected members of the radiated pairs and the single animals receiving only 27 roentgens showed no gross signs of radiation injury. However, about 4 weeks following radiation both members of the six pairs in Group I started to lose their hair. Such a pair is shown in Fig. 2. The epilation was observed simultaneously but was first much more marked in the protected members. Rough estimation of the degree of epilation (1 to 4+) was made and the tabulation of these estimations for the 6 pairs, Fig. 3, gives the course of the epilation in the 2 animals. The protected animals were affected to a greater degree but for a shorter time than the unprotected members. Neither the single controls, receiving 27 roentgens, nor the non-radiated parabionts of Group II showed any sign of epilation. The single controls receiving 900 roentgens died within one week after radiation. The protection afforded the radiated parabiont will be discussed in a later communication.


FIG 2.
Parabiotic rats showing epilation of both (A) irradiated and (B) non-irradiated members.

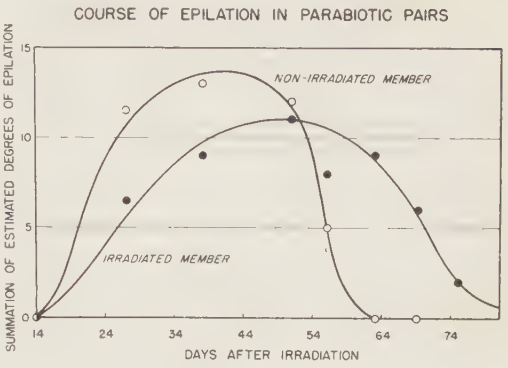


FIG. 3.

*Discussion and Conclusion.* Parabiotically united rats influence their partners by the mixing of their body fluids through their common circulation. Therefore, an effect such as epilation must result from the transfer of material from one member to the other. Whether the epilation represents the passage of physiologically active material from the radiated to the non-radiated or is a manifestation of loss of material from the non-irradiated to the irradiated is not demonstrated.

[10] Van Dyke, D. C., Huff, R. L., Evans, H. M., *Stanford Med. Bull.*, 1948, **6**, 271.

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# Effect of Electroconvulsive Shocks on Nest Building in the Male Albino Rat.* (17403)

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In an investigation of the effect of electroconvulsive shock on maternal behavior, Rosvold[1] studied the change in nest building activity in the female albino rat. His data show definite interference with nest building activity, varying in degree with the length of time after parturition that the series of convulsive shocks is introduced.

Since Kinder[2] has shown that males build nests when environmental temperatures are low, it is possible to test the effects of electroconvulsive shocks on nesting activity in animals free from the influence of the physiological conditions due to pregnancy and maternity. Should the shock disrupt the nest building of males, it could be argued first, that shock influences some factor common to both sexes rather than one specific to the female, and second, that the effect in the females is not the result of the combined stress of the convulsive shock and maternity.

*Procedure.* Twenty-one adult male albino rats approximately 100 days old were selected from the Stanford laboratory stock. Twelve of these were shocked each day for 9 days, after which they were moved into a relatively cold attic room (diurnal range, 52°-75°F; usually below 65°F) for observation of their nest building activity. Nine, not shocked but otherwise treated like the experimental animals, served as controls. Each male was placed in a cage similar to those used for the females of Rosvold's[1] study. Shavings and paper were provided for nesting. A tenth shock was given about 24 hours after the rats had been in the attic. Thereafter they were undisturbed except for routine care and observation, and the nests were left intact.

A five point scale was used to rate their nests. The points were defined as 0 for no use of nesting materials, 1 if the paper had been moved to a preferred sleeping place, 2 if all the paper had been gathered, 3 if a bowl had been formed with paper, and 4 if the best possible nest had been built of shavings and all of the paper.

The electroconvulsive shocks were induced by administering 45 milliamperes for 0.2 second through alligator clips attached to the rat's ears. This strength of current invariably induced a complete tonic-clonic convulsion.

*Results.* During the *first* day in the cold room the temperature did not go above 64°F which, according to Kinder,[2] should have afforded ample thermal incentive for nest building. Table I gives the ratings assigned to the nests on each observation day in the attic room. The control animals began nesting immediately, and after 6 hours 2 had built nests that were rated as 1, one rated as 2, two as 3, four as 4. Not one of the shocked animals (having received 9 shocks) built a nest on the first day in the attic room.

On the *second* day the shocked males experienced the tenth convulsion while the controls were removed from the cages for a similar length of time. On this day the control group improved their nests with the result that 6 were rated 4, two rated 3, and one, 2. Most of the shocked animals did very little nest building. One gathered some paper into a preferred corner and was rated 1, and another built a nest base which was rated 2. Twenty-five university students taking a course in comparative psychology distinguished between the experimental and control groups very definitely, giving the shocked group less than 15% of a perfect score and the control group more than 80% of a perfect

* Financed from a Scottish Rite Research Fund administered at Stanford University by Calvin P. Stone.

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1 Rosvold, H. E., *J. Comp. Physiol. Psychol.*, 1949, **42**, 118.

2 Kinder, E. F., *J. Exp. Zool.*, 1927, **47**, 117.

TABLE I.
Ratings of Nests on Each Day in the Attic Room.
(Rating of shocked rats 1-7 and controls 1-4 was discontinued on the 10th day, post shock).

| Day in attic | Shock | Daily ratings on a scale of 0-4 | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Shocked animals (Nos.1-12) | | | | | | | | | | | | Control animals (Nos. 1-9) | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | 9th | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 4 | 3 | 1 | 3 | 2 | 4 | 4 |
| 2 | 10th | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 3 | 2 | 4 | 3 | 4 | 4 |
| 3 | 0 | 1 | 0 | 1 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 3 | 4 | 4 | 4 | 2 | 3 | 4 | 4 | 4 |
| 4 | 0 | 1 | 0 | 0 | 3 | 0 | 1 | 2 | 1 | 0 | 1 | 1 | 1 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 4 | 4 |
| 5 | 0 | 1 | 1 | 0 | 3 | 0 | 1 | 3 | 0 | 1 | 1 | 2 | 1 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 4 | 4 |
| 6 | 0 | 2 | 1 | 2 | 4 | 1 | 1 | 3 | 1 | 0 | 2 | 3 | 2 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 3 | 4 |
| 7 | 0 | 2 | 2 | 3 | 4 | 1 | 2 | 4 | 1 | 0 | 2 | 3 | 2 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 3 | 4 |
| 8 | 0 | 4 | 3 | 3 | 4 | 2 | 2 | 4 | 2 | 1 | 3 | 4 | 2 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 3 | 4 |
| 9 | 0 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 2 | 1 | 3 | 3 | 2 | 4 | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 3 |
| 10 | 0 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 2 | 2 | 3 | 4 | 3 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 4 | 4 |
| 11 | 0 | – | – | – | – | – | – | – | 1 | 0 | 1 | 2 | 2 | – | – | – | – | 3 | 3 | 3 | 3 | 4 |
| 12 | 0 | – | – | – | – | – | – | – | 1 | 3 | 2 | 2 | 4 | – | – | – | – | 3 | 3 | 4 | 3 | 3 |
| 13 | 0 | – | – | – | – | – | – | – | 3 | 3 | 3 | 4 | 4 | – | – | – | – | 4 | 2 | 4 | 3 | 4 |
| 14 | 0 | – | – | – | – | – | – | – | 2 | 2 | 3 | 3 | 4 | – | – | – | – | 3 | 2 | 3 | 3 | 3 |
| 15 | 0 | – | – | – | – | – | – | – | 2 | 3 | 2 | 3 | 4 | – | – | – | – | 4 | 3 | 3 | 3 | 3 |
| 16 | 0 | – | – | – | – | – | – | – | 1 | 2 | 1 | 3 | 4 | – | – | – | – | 3 | 3 | 2 | 3 | 3 |
| 17 | 0 | – | – | – | – | – | – | – | 4 | 3 | 2 | 3 | 4 | – | – | – | – | 4 | 3 | 4 | 3 | 4 |
| 18 | 0 | – | – | – | – | – | – | – | 3 | 3 | 2 | 3 | 4 | – | – | – | – | 3 | 2 | 4 | 3 | 3 |
| 19 | 0 | – | – | – | – | – | – | – | 1 | 3 | 2 | 3 | 4 | – | – | – | – | 4 | 3 | 4 | 3 | 4 |

score.

No further shocks were administered. As can be seen in Fig. 1, shocked animals gradually began to build better nests, but it was not until the fifth day after the last shock (seventh day in the attic room) that the nests of the convulsed animals began to approach those of the control rats in structure and composition. By the seventh day, post shock, the nests of 4 of the experimental animals could not be distinguished from those of the control group, but those of the other 8 were slightly inferior. By the tenth day there were only minor differences on the part of a few of the shocked rats.

On the tenth day, post shock, the nests of 5 animals from each group were removed and fresh nesting materials provided. Differences between shocked and non-shocked animals showed up again. By the following day (the eleventh day) the control animals had rebuilt good nests while the convulsed animals had built nests which deserved ratings no better than 2. Improvement was rapid, however, so that by 48 hours later, nests which were rated 3 and 4 were built. This rating surpassed their previous highest rating and nearly equalled that of control group. Thereafter no constant differences were found.

Rating of 5 of the shocked rats and five of the controls was continued until the nineteenth day, post shock, when the same group of psychology students were told that the positions of the animals had been changed. Using the same criteria for rating, they were unable to distinguish between the two groups.

*Discussion.* The study reported in this paper combines the findings of 2 experimenters using the same procedures. The behavior of the shocked animals in both instances was so identical as to warrant reporting them as one study. The fact that the same results were obtained by 2 experimenters working one year apart lends support to the validity of the findings. We may conclude that electroconvulsive shocks disturb nest building activity in the male albino rat in an environment in which the diurnal range of temperatures is from 52°-75°F, and usually below 65°F. Furthermore, the disruptive effects of convulsive shocks on nest building activity of the male rat demonstrates that the effects in the female described by Rosvold[1] may be common to both sexes, and that they are not necessarily the result of the combined factors of shock and maternity.

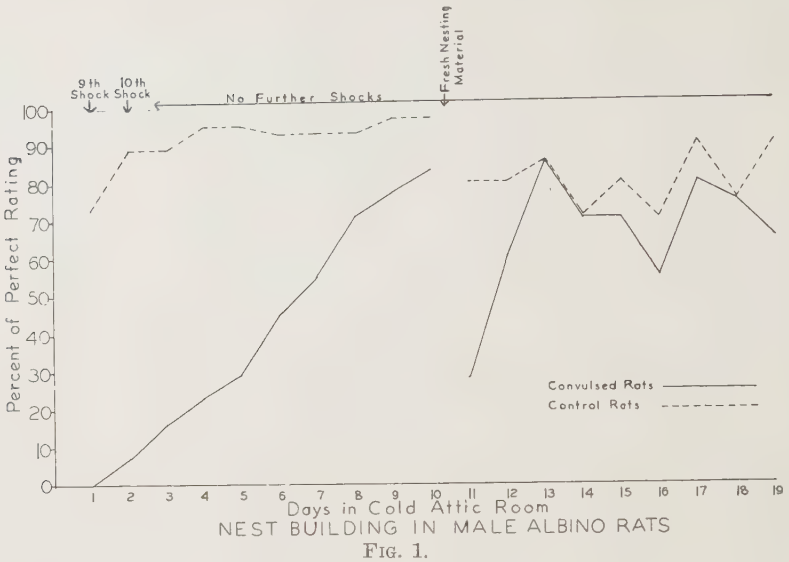


FIG. 1.

Beach[3] has stated that adequate functioning of innately organized reproductive behavior depends on the organism being under the influence of certain hormones at critical periods. Unless the hormones are present in sufficient strength at the right time, stimuli specific to an innately organized pattern of behavior have no effect. Rosvold[4] suggested that the inability of the parturient mother to respond to the paper and shavings as nesting materials may have been due to the fact that the innate organization for these responses had not been sensitized by the appropriate hormones. He implied that the hormones affected were related to the pregnancy-lactation cycle. Such an hypothesis, without qualification, is no longer tenable.

*Summary.* Twelve adult male albino rats were shocked once a day for 9 days after which they were placed in a relatively cold attic room and supplied with nest building materials. Nine, not shocked but otherwise treated like the others, served as controls. In the early post shock period the convulsed animals made no attempt at nest building whereas their controls built excellent nests. There was gradual recovery so that by the tenth day post shock, the convulsed animals built nests equal to those of the control rats.

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[3] Beach, F. A., *Hormones and Behavior,* New York: Paul Hoeber, Inc., 1948, pp. 368.

[4] Rosvold, H. E., *J. Comp. Physiol. Psychol.,* 1949, **42**, 207.

## Hypotensive Action of Influenza A Virus on Rats.* (17404)

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The frequent clinical observations of cardiovascular disturbances complicating influenza infection[1,2] prompted us to undertake an experimental investigation of the toxic effect

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[1] Findland, M., *et al., Am. J. Med. Sc.,* 1945. **209**, 455.

of the virus on the cardio-vascular system of the host. During a study on the cardiac output of ferrets infected by the PR8 strain of influenza virus[3] arterial hypotension was occasionally noticed. Further observations disclosed that this phenomenon was invariably produced when albino rats were given intravenous injections of the virus. A series of experiments were then carried out to determine the nature of this factor in our preparation.

*Preparation of the virus.* A PR8 strain of influenza virus which was lethal for mice on intravenous injection[4] was originally obtained from Dr. W. Henle and maintained by passages in embryonated eggs by the usual procedures of allantoic sac inoculation, incubation and harvest.[5] The pooled, bacteria-free allantoic fluid was centrifuged at 1050 g to remove the debris and then purified by adsorption and elution from chick red blood cells.[6] The eluate was then centrifuged at 20,000 g for one hour and the precipitate was suspended in 0.1 M phosphate buffer solution of pH 7.0. A final centrifugation at 1050 g removed larger particles. In two experiments, the material was further purified by methanol precipitation according to the method of Cox *et al.*[7] The final product has an average hemagglutination titer[8] of 64,000 per mg of nitrogen, and its infectivity titer for chick embryo was $10^{-13.4}$ g. The extent to which our material was free from non-viral proteins was about the same as the preparations of other authors working on the purification of influenza virus.[9,10]

*Preparation of the animals.* 250-300 g male albino rats (Harlan strain) were anesthetized with sodium nembutal and the trachea was cannulated. Arterial blood pressure was obtained by direct cannulation of the common carotid artery and registered by mercury manometer writing on a kymographic drum. One ml of the virus suspension per 100 g of body weight was injected into the jugular vein at the rate of one ml per minute. The blood pressure was then observed for the next 30 minutes.

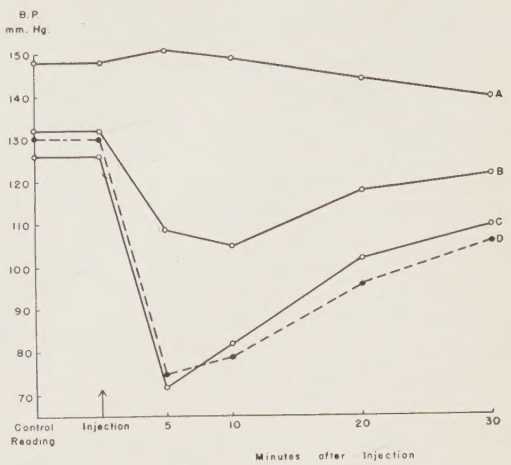


FIG. 1.

Hypotensive action of virus prepared by different purification procedures and that of normal allantoic fluid.

A—Inj. of conc. normal allantoic fluid.

B—Inj. of virus conc. by adsorption and elution from chick rbc.

C—Inj. of virus conc. by adsorption and elution from chick rbc. plus high speed centrifugation.

D—Inj. of virus conc. by adsorption and elution from chick rbc. plus high speed centrifugation and methanol precipitation.

*Results.* The data of the experiments can be summarized in the following categories:

1. Allantoic fluid of normal 11-day-old chick embryos was harvested, purified and concentrated by the same procedures used for the virus. Injection of this control material did not alter the blood pressure (Fig. 1, A).

2. The hypotensive factor seemed to be associated with the virus particles since it was similarly adsorbed and eluted from chicken red blood cells sedimented by high speed centrifugation and precipitated by methanol (Fig. 1, B, C, D). When the infectious allantoic fluid was centrifuged at 20,000 g, the precipitate resuspended in buffer solution gave

2 Andrews, C. L., *J. Med. Soc. N. J.*, 1940, **37**, 16.

3 Kempf, J. E., and Chang, H. T., submitted for publication.

4 Henle, G., and Henle, W., *J. Exp. Med.*, 1946, **84**, 623.

5 *Diagnostic Procedures for Viruses and Rickettsial Diseases, Am. Pub. Health Assn.*, 1948, 99.

6 Hare, R., *et al., Canad. J. Pub. Health*, 1946, **37**, 284.

7 Cox, H. R., *J. Immunol.*, 1947, **56**, 148.

8 Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

9 Sharp, A. R., *J. Immunol.*, 1944, **48**, 129.

10 Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 255.

TABLE I.

Effect of Heat on Hemagglutination, Chick Embryo Infectivity, Mouse Toxicity and Hypotensive Factor of the Concentrated Virus Preparation.

| Treatment of the purified virus | Hemagglutination titer | Infectivity titer | Mouse toxicity* | % of drop in B.P. after inj. into rats† |
|---|---|---|---|---|
| Unheated | 1:16000 | $10^{-10}$ | 1:16 | 42.9 |
| Heated at 56°C for 4 hr | 1:8000 | $10^{-1}$ | 1:4 | 28.2 |
| 8 hr | 1:200 | 0 | 1:2 | 33.6 |
| 12 hr | 0 | 0 | 0 | 18.2 |
| 16 hr | 0 | 0 | 0 | 5.4‡ |

* Kills 4 out of 6 in 48 hr.

† % of drop in B.P. $= \frac{\text{Max. drop of B.P. in mm Hg}}{\text{Control B.P. reading before inj.}} \times 100.$

‡ This figure is equivalent to spontaneous variation in B.P. during the period of observation on control animals.

TABLE II.

Effect of Specific Anti-Serum on Neutralization of Hypotensive Factor of the Virus Preparation.

| Preparations injected into rats | No. of rats used | Max. drop in B.P. during first 30 min. after inj.* | % drop in B.P. after inj. |
|---|---|---|---|
| A. Infectious allantoic fluid neutralized first and then concentrated | 6 | 0 mm Hg | 0 |
| B. Purified virus plus specific immune ferret serum | 5 | 17 | 11.2 |
| C. Purified virus plus normal ferret serum | 6 | 49 | 32.9† |

* Initial B. P. for all these animals during control period before injection (average) $= 150 \pm 2$ mm Hg.

† Difference between values for B and C is highly significant statistically. (P value less than 0.01).

a maximal decline of 40 per cent in blood pressure while the supernatant of the same preparation was entirely without effect.

3. The hypotensive factor was found to be relatively heat resistant being destroyed only after heating at 56° C for 16 hours (Table I). The hemagglutinins, infectivity for chick embryos and toxicity for mice were all inactivated by heating at the same temperature for shorter periods of time respectively.

4. Ferret immune serum titering 1:12500 by the hemagglutination inhibition test was produced by injection of infected mouse or ferret lung tissue suspension. Addition of this serum to pooled allantoic fluid of infected chick embryos resulted in complete neutralization of the virus as indicated by the hemagglutination and chick embryo neutralization tests. This neutralized mixture was then treated by adsorption and elution from chick red blood cells plus high speed centrifugation. Injection of the resulting preparation showed

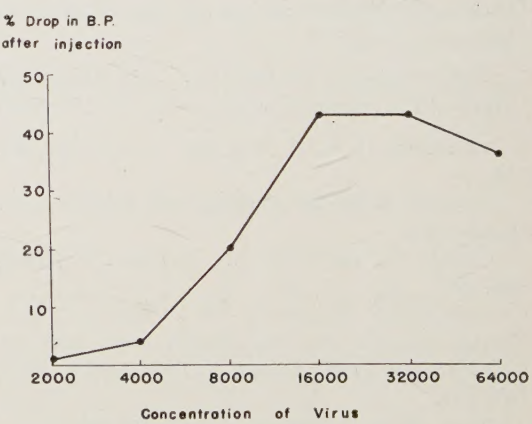


FIG. 2.

Relationship between concentration of the virus (shown by hemagglutination titer) and hypotensive response.

the loss of the hypotensive factor indicating specific neutralization of the factor (Table II, A).

To prove further that the hypotensive factor was a fraction of the virus particles, the concentrated and purified preparation was mixed directly with the immune serum. Injection of the mixture into rats showed a significant reduction of the hypotensive effect in comparison with the control material to which normal ferret serum was added (Table II, B, C).

6. To elicit the hypotensive response a threshold concentration of virus with hemagglutination titer of 1:8000 was required. Concentrations of virus with titer between 1:16,000 and 1:64,000 elicited about the same response (Fig. 2).

*Conclusion.* These observations indicate that a fraction of the PR8 strain of influenza virus possesses the toxic property of lowering arterial blood pressure in rats.

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## Effect of Congo Red on the "MM" Virus. (17405)

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Conflicting reports on the effect of trypan red on the "MM"[1] neurotropic virus have appeared in the literature. Wood and Rusoff[2] reported this compound along with two other acid dyes, Congo red and brilliant vital red, to be definitely effective against the virus. Using the intraperitoneal injection route followed by intraperitoneal inoculation of the virus, these workers found an apparent protection which was overcome by inoculation of the virus in too high concentration.

Hammon *et al.*[3] have reported the failure of trypan red to protect against the "MM" virus when the dye was injected subcutaneously and the virus inoculated by intraperitoneal route. These authors interpreted the results of Wood and Rusoff as being due to a non specific protection such as afforded by various inert substances when injected previous to the inoculation of infectious agents by the same route.

Since the effect of Congo red had been reported for only one concentration of "MM" virus it was thought desirable to determine the activity over a range of dilutions.

In one series of experiments Congo red was injected intraperitoneally into 12 g mice and in a second series the dye was administered by stomach tube. As a matter of interest trypan red was included in 2 of the experiments. All virus inoculations were by the intraperitoneal route, serial dilutions being made from frozen stock material. A range (10-fold) of infecting dilutions was employed so as to permit calculation of LD50 values by the method of Reed and Muench.[4] Unless otherwise specified, dilutions of $10^{-6}$ through $10^{-9}$ were used for controls and $10^{-5}$ through $10^{-8}$ for test groups, 6 mice being used per dilution.

In all experiments calling for the injection of dye the schedule employed was that used by Wood and Rusoff. A 1% solution was injected in 0.1 cc amounts on each of 3 days and the virus was administered one day after the last injection.

For the experiments in which the dyes were given by stomach tube the schedule called for the feeding of 0.5 ml of a 1% solution twice daily for 9 feedings with the virus inoculation following the fourth feed-

1 Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

2 Wood, H. G., and Rusoff, I. I., *J. Exp. Med.*, 1945, **82**, 297.

3 Hammon, W. McD., Aird, R. B., and Sather, Gladys, PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 511.

4 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Effect of Intraperitoneally Injected Dye on "MM" Virus.

| | Total No. of mice | Control, $LD_{50}$ | Test, $LD_{50}$ | $LD_{50}$ Difference |
|---|---|---|---|---|
| Congo red | 54 | 8.2 | 6.0* | —2.2 |
| | 100 | 7.0† | 7.1‡ | +0.1 |
| | 48 | 7.5 | >5.0 | >—2.5 |
| Trypan red | 48 | 7.0 | 7.7 | +0.7 |

* Dilution range 10–4 through 10–8.
† Dilution range 10–5 through 10–9, 10 mice per dilution.
‡ Dilution range 10–4 through 10–8, 10 mice per dilution.

TABLE II.
Effect of Orally Administered Dye on "MM" Virus.

| | Total No. of mice | Control, $LD_{50}$ | Test, $LD_{50}$ | $LD_{50}$ Difference |
|---|---|---|---|---|
| Congo red | 48 | 7.5 | 5.2 | —2.3 |
| | 48 | 8.2 | 5.8 | —2.4 |
| Trypan red | 48 | 7.0 | 5.6 | —1.4 |

ing. It was felt that two daily feedings would roughly approximate the amounts administered in drug diets.

The results of experiments in which the dye and the virus were administered by intraperitoneal injection are shown in Table I. It can readily be seen that any protection index based on the LD50 differences recorded for Congo red will have a wide range and the validity of a claim for protective action would be open to serious question. The erratic results presented are typical of other experiments and it is not felt that a truly protective compound would be subject to such variable results.

Table II shows the effect of dye administered orally, the virus being given by intraperitoneal injection. While the apparent protection may be due to non specific factors, such as handling of the mice, it is of interest to note that such apparent protection may be evoked by administration of the dye through a route other than that used for the virus injection.

Calculation of the LD50 standard errors would undoubtedly show that little confidence could be placed in the LD50 differences recorded and it is not felt that any of the differences are indicative of significant protection. In considering the work of Wood and Rusoff it would not seem that a really effective compound would be active against one concentration of virus only and we feel that the expression "apparent protection" is more accurate.

*Conclusions.* Congo red was found to stimulate an apparent protection against the "MM" virus when administered by a route other than that used for virus inoculation. It is not felt that the degree of protection is significant.

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